Laser Microdissection Separation of Pure Spermatozoa Populations from Mixed Cell Samples for Forensic DNA Analysis

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Abstract

Short Tandem Repeat (STR) analysis is a valuable tool in identifying the source of biological stains, particularly from the investigation of sexual assault crimes. Difficulties in analysis arise primarily in the interpretation of mixed genotypes when cell separation of the sexual assailant's sperm from the victim's cells is incomplete. The forensic community continues to seek improvements in cell separation methods from mixtures for DNA typing. The feasibility of

applying laser microdissection (LMD) technology to precisely separate sexual assault cell mixtures by visual inspection coupled with laser dissection was assessed through a) evaluation of various histological stains for use with LMD and downstream DNA analysis, b) identification of an appropriate DNA isolation method for LMD collected cells, c) quantitative analysis of DNA from both sperm and epithelial cells collected by LMD, d) Short tandem repeat typing from LMD separated sperm cells from mixtures of semen and female buccal epithelial cells using both standard and low copy number DNA analysis, and e) a comparative study between the LMD method and the preferential lysis method for separation of sperm from epithelial cells at various mixture ratios for low copy number analysis. Also, four genuine case samples were examined using LMD. The results of these analyses indicate that: 1) Both hematoxylin/eosin and nuclear fast red staining performed well in their ability to differentiate sperm and epithelial cells without interfering with downstream analysis; 2) Both QIAamp® and Lyse-N-Go™ methods were useful for recovery of DNA from LMD collected sperm cells; 3) DNA extraction yields from LMD collected cells ranged from 12-45%; 4) LMD separation provided clear STR profiles of the male donor with effective separation from the epithelial cell component, and 5) LMD outperformed preferential lysis in separation of sperm from increasing proportions of epithelial cells. This report describes an efficient, low-manipulation laser microdissection method for the efficient separation of spermatozoa from two-donor sperm/epithelial cell mixtures for DNA analysis.

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Executive Summary

Forensic DNA analysis plays a central role in the human identification of perpetrators involved in sexual assault crimes. Spermatozoa are typically the biological material of interest in rape evidence, however, this type of evidence is often commingled with cells from the alleged victim complicating the genotyping of the assailant. Unfortunately, mixtures can be difficult to detect and decipher without proper training, as one must understand how single source and mixed samples behave in addition to de-convoluting mixed DNA profiles against a background of biological and technological artifacts (Butler 2006). Compounding the challenge of genotyping these types of profiles are the theoretical aspects of statistical calculations for mixture interpretation, which are complex and difficult to convey to a jury. Therefore, in physical evidence containing semen, the successful separation of spermatozoa from other cells is imperative for unambiguous genotyping of the assailant.

In 1985, Gill et al. developed a method to selectively remove epithelial cells from a sperm and epithelial cell mixture. The method gently lyses the non-sperm cells releasing the DNA into the supernatant while sperm cells remain intact. The free-floating DNA is then removed by several washes leaving a sperm cell pellet. This method of preferential lysis has been considered the primary standard of practice employed in forensic laboratories for over 20 years. Although preferential lysis can generally provide a fraction enriched with sperm the separation is often not complete as this traditional method at times is inefficient in removing soluble DNA from the cell pellet. This problem of incomplete separation is frequently an issue with samples containing large numbers of victim's epithelial cells. At the time this method was first developed, the

technology to detect various genetic markers were not nearly as sensitive as it has become today. Perhaps carryover of epithelial cell DNA into the sperm fraction was considered only a minor nuance at that time, but today very minute amounts of DNA can be detected and even small amounts of contaminating DNA can make genotyping challenging. In addition the preferential lysis method is time consuming, and requires multiple sample manipulation that can lead to loss of critical sperm. More recently the forensic community has explored methods to improve the separation and detection of sperm for sexual assault cases, however, the high degree of precision required for a pure separation has previously not been available.

An alternative to a chemical separation, such as the preferential lysis method, is separation by direct physical selection of the target cells from a mixture. Recent advances in microscopic instrumentation now permit direct visualization, dissection and recovery of specific cells and tissue from microscope slides using laser illumination; this approach is called laser microdissection. We have developed a method using laser microdissection technology to selectively separate pure populations of sperm from epithelial cells for STR analysis. Three practical applications were developed that could improve the outcome of sexual assault case analysis as well as increase the throughput of difficult evidence samples. These methods include: i) Laser microdissection separation of spermatozoa from epithelial cells for standard STR analysis ii) Laser microdissection recovery and separation of minute numbers of spermatozoa from epithelial cells for low copy number STR analysis and iii) Quantiation of DNA using LMD cell recovery. The development of each method is summarized below:

i) LMD was evaluated and a method developed to separate pure populations of

sperm cells from semen/epithelial cell mixtures compatible with DNA analysis. With any new technology introduced into the legal arena, validation studies are critical but also time consuming. We therefore found it important to incorporate an LMD protocol with commonly used upstream and downstream methods used in forensic biology laboratories, which we refer to as "standard" analysis in this report. By avoiding manipulating these well-established DNA methods, the LMD method described can be better implemented into practical use. A series of three experiments were performed to assess LMD technology. First, the effect of various histological stains on downstream analysis from LMD stained cells was examined. Second, DNA isolation methods were compared when applied to LMD collected cells. Finally, the separation capability of LMD to recover sperm cells from epithelial cells in a stained mixed specimen was determined.

To ascertain an appropriate histological stain for LMD recovery several common stains were tested for their utility in sperm and epithelial cell identification and their effects on downstream DNA analysis: hematoxylin/eosin (H&E), nuclear fast red (NFR), nuclear fast red/picroindigocarmine (CTS, also known as "Christmas tree stain"), methyl green (MG), Wright's stain (WRT) and acridine orange (AO), and SYBR 14/propidium iodide. The H&E staining using abbreviated incubation times and NRF were found to provide good discrimination of sperm cells without affecting further downstream analysis.

Unique challenges were faced when developing and determining which DNA isolation method was appropriate for LMD cells. When confronted with molecular DNA analysis of histologically stained cells, a method that could remove Taq inhibitors from the sample would be advantageous. At the same time, conservation of DNA from the

recovered cells was crucial. We evaluated the Qiagen QIAamp®, Lyse-N-Go™, and MicroLYSIS® products for use with LMD collected cells. Both the QIAamp® and Lyse-N-Go™ methods were found to be effective with the DNA isolation of sperm cells. Lyse-N-Go™ had the advantage of being a rapid, low-manipulation method while remaining inexpensive to use. QIAamp® has the advantage of obtaining a purified DNA sample that could be useful in forensic specimens containing inhibitors.

To evaluate the separation capability of LMD, mixed cell samples made from semen and buccal swabs were examined. Collections of 75, 150 and 300 stained sperm cells were separated by LMD from mixtures followed by STR analysis. In all samples tested, the semen donors' genotypes were detected with the absence of any non-shared alleles from the female buccal cell donors. The limit of sensitivity using standard PCR protocols began to diminish below the "150 sperm cell" samples, but partial profiles could still be obtained from the "75 sperm cell" samples. Results obtained after additional PCR cycles were applied the amplified products further demonstrated that pure populations of sperm were recovered from semen/epithelial cell mixtures as no female DNA was detected in these samples. The method we developed therefore would be appropriate for use with the LMD collection of over 75 sperm cells when employing typical forensic PCR conditions.

ii) An area of research of great interest in the forensic community is the capacity of obtaining genotypes from minute amounts of DNA. Low copy number (LCN) DNA analysis typically refers to the testing of less than ~100pg of input DNA into a PCR reaction and is a technique sensitive enough to analyze just a few cells One of the challenges of LCN analysis is generating a reliable DNA profile as this type of analysis

typically is accompanied by artifacts that can even further complicate the analysis when de-convoluting genuine DNA mixtures and in deciphering if the observed peaks are PCR related or true alleles in biological samples from either single or multiple sources. In addition, the technique is so sensitive that exogenous DNA unrelated to a crime, such as transfer of DNA from previous handlers of an object in question or unintentional transfer of DNA from technicians may be detected.

In addition to the LMD capability of excluding unwanted cells in a sample, it may also be useful for avoiding free floating exogenous DNA which is problematic in LCN work. In this study, LMD was tested for the recovery of minute numbers of sperm cells from mixtures for LCN DNA analysis. A series of three experiments was performed to assess the functionality of LMD collection for LCN analysis. First, the minimum number of LMD-collected cells necessary for STR typing was determined using our LCN protocols. Second, a comparative study was performed on the LMD method vs. the preferential lysis method for the separation of sperm from increasing proportions of female epithelial cells for LCN analysis. Finally, four sexual assault case studies were examined using LMD and LCN analysis.

To establish the minimum number of sperm cells detected by STR amplification using our LCN analysis conditions, sperm cells were recovered from mixtures of semen and female buccal cells followed by DNA analysis. Mixtures of buccal and sperm cells were created to be equivalent to one oral swab and 1µl of semen. Collections of 5, 10, 20, 40 and 80 sperm cells were separated by LMD from each mixture and analyzed for STR loci using 34-cycle conditions (6 more cycles than standard conditions). Nearly all loci tested could be detected from samples containing 20-80 sperm cells. Below 20 cells

allelic dropout was pronounced; however, clear partial profiles could still be obtained from as few as 5 sperm cells.

To further evaluate the separation and sensitivity of the LMD method, sperm from specific ratios of sperm and epithelial cell mixtures were separated using both the LMD and preferential lysis method. Cell mixtures were created at 1:5, 1:10, 1:20, 1:40, 1:80 and 1:160 (sperm: epithelial). Portions of each mixture were processed by LMD separation and the preferential lysis separation for comparison and analyzed for STR loci using LCN analysis. Although each method performed similarly in detection and separation of the male component from the 1:5 and 1:10 mixtures, the LMD method outperformed the preferential lysis method as the epithelial cell component continued to increase. In addition, the LMD method produced sperm donor profiles without any detectable DNA from the female donor.

With the success of our controlled experiments it became clear that the final test of the LMD method would be to examine real sexual assault evidence samples. Forensic specimens are unique in that they are exposed to an unlimited variety of conditions that may impact the extent and quality of DNA results. We obtained sperm positive case samples that had previously been analyzed by a police laboratory, which we re-analyzed by LMD. The samples with high densities of epithelial cells proved to be challenging; however, in the four cases examined our results produced similar results in one case, improved results in two cases and less information in one case than when originally tested by the crime laboratory. Since our sample set was limited, we recommend that a much larger pool of casework be examined representing a broader variety of sexual

assault evidence. A large comparative case study examination can help define criteria for evidence samples that may benefit from LMD separation over the traditional PL method.

iii) Determination of the quantity of DNA in a sample is important for most PCR-based assays to ensure the reaction is performing optimally. The LMD collection process may provide an indirect estimate of DNA obtained without consuming any additional DNA extract. Since the number of cells can be counted during LMD collection the amount of starting DNA material in the cells can be estimated. However, loss of DNA during the isolation procedure must also be considered and some measure of the extraction efficiency can then be used to estimate the amount of DNA in the final extract prior to PCR.

In this study we performed quantitative analysis on QIAamp® DNA extracts from LMD cells to determine the amount of loss associated with the extraction process and the feasibility of using LMD collection as a quantitative measure of DNA for PCR. Collections of 5, 10, 20, 40, 80, 150, and 300 sperm cells were recovered by LMD from the semen smears. Collections of 2, 5, 10, 20, 40, 80, and 150 epithelial cells were also collected by LMD from buccal cell smears. Two methods were used to evaluate yield. The first was by direct quantification of DNA by a real-time qPCR. The second method was using total RFU signal of STR results to measure relative amounts of PCR product to a standard curve.

The highest yields of DNA were from the epithelial cells where approximately 1/2 to 1/3 of the DNA was recovered, whereas DNA obtained from sperm cells was less than 1/4 of the original starting material. A dilution series of a known quantity of DNA was

also processed through the same QIAamp® extraction procedure and then re-quantified leading to a similar loss in yield, which indicates that the DNA loss occurred during the extraction process and not due to a failure of LMD cell recovery into the collection tube. Despite the apparently low yields, the importance of this study was that the efficiency of the QIAamp® extraction remained relatively consistent across a range of amounts of starting material acquired by LMD. Therefore, during the collection process LMD can also be used to estimate the quantity of DNA available for PCR.

The main body of our report describes in detail the development of an efficient, low-manipulation laser microdissection method for the effective separation of spermatozoa from two-donor sperm/epithelial cell mixtures for DNA analysis. LMD could assist forensic scientists in analyzing sexual assault mixtures that previously could not be effectively separated using conventional means. In addition, LMD could be the tool of choice for collection of minute numbers of sperm in mixtures for low copy number analysis.

Introduction

Sexual violence is a serious problem that affects millions of people every year in the United States [Center for Disease Control, 2006]. The use of forensic DNA analysis has become a crucial tool in identifying perpetrators of sexual assault crimes. Typically the biological evidence of interest is semen, however, this evidence is commonly deposited along with the victim's cells from a vaginal, rectal, or oral cavity complicating the DNA genotyping of the assailant.

Biological stains from two or more individuals that cannot be separated result in a mixed genotype. The data then requires a multifaceted statistical analysis with an interpretation involving some subjectivity by the analyst. Mixture interpretation is complicated by a number of technical factors including, but not limited to, allele overlap, stochastic fluctuation, low quantity of DNA, degraded template, three-allele patterns, microvarients [Ladd et. al., 2001] and strand slippage stutter [Moxon et al., 1999]. Typically, mixed genotype analysis is time consuming for the forensic practitioner and challenging for an expert witness to successfully convey to a jury, potentially lessening the power of the evidence.

Success of this project would reduce the need for mixture interpretation in many cases by addressing the problem early in the line of evidence analysis, during sperm identification. The development of a method capable of fully separating spermatozoa from epithelial cells before DNA analysis would result in more readily interpretable typing patterns, thus improving the chances for a successful individualization. As a

result, testimony in these cases would be more straightforward and better understood by the judicial system resulting in the more effective adjudication of sex crimes.

The preferential lysis method [Yoshida et al., 1995; Gill et al., 1985] has been the forensic standard for separating sperm cells from epithelial cells. This method utilizes cell-specific differences in membrane chemical composition by first lysing the non-sperm cells without disrupting the sperm cells, then washing away any residual exogenous DNA from the intact sperm cells. Although this method can generally provide two cellular fractions, one comprising of sperm cell DNA and the other of non-sperm DNA, the separation is not always complete. There may be carryover from one cell fraction to another making genotype interpretation and statistical analysis challenging. Additional limitations to this technique are the premature lysis and loss of sperm cells in the first digestion and the multiple liquid transfers and washing steps that reduce cell recovery. The development of a method capable of fully separating pure populations of spermatozoa from epithelial cells while conserving sample would enable analysts to interpret DNA typing patterns with less difficulty.

Flow cytometry was introduced as a superior method to separate sperm cells from vaginal epithelial cells [Schoell et al., 1999] in the late 1990s. This fluorescence-activated cell sorting (FACS) approach relies on differences in the cell size, shape, surface phenotype, cytoplasm and DNA content. It has been reported that flow cytometry has improved sensitivity relative to preferential lysis in identifying male DNA in a mixture [Schoell et al., 1999]. However, this high sensitivity requires a low concentration of cellular debris in the vaginal lavage. FACS was also successful in separating sperm from a mixture as low as 1:160 sperm/vaginal cell ratio, which provides superior resolution

over the preferential lysis method. Unfortunately, FACS also requires a large number of target cells as starting material (6000 sperm in 1 million vaginal cells). In addition, the starting cell suspension requires a lengthy, multi-step florescent immunostaining process before initiating cytometry. There are no reports applying FACS to analysis of non-clinical samples, such as samples from post-mortem collection or crime scene samples which could be a problem as larger amounts of released DNA from degraded cells would be expected in these types of samples.

A microchip-based sperm and epithelial cell separation method utilizes the differential physical properties of cells that result in settling of the epithelial cells to the bottom of a reservoir and subsequent adherence to the glass substrate [Horsman et al., 2005]. The flow rate can be used to separate the sperm cells from the epithelial cell-containing mixture. Semen donor profiles using AmpF*l*STR COfiler amplification were clearly obtained from the male fractions, but the presence of female DNA in the sperm fraction was evident - most likely to epithelial cell DNA free floating in the sample. This separation procedure can be performed under 30 minutes and has the potential of being automated. However, the sperm recovery using gravity-driven flow was less than 5% and only increased to ~25% by extending the flow time to 70 minutes. The authors suggest that using a negative pressure flow instead may improve this yield, although, this method may not be suited for samples with minute numbers of sperm.

Membrane filtration was another separation method introduced as an alternative to the preferential lysis method [Chen el al., 1998]. This filtration method was developed to cleanly separate spermatozoa from epithelial cells based upon differences in size and shape. This is a very simple and rapid technique with separation based on choice of the

appropriate filter pore size and either low speed centrifugation or vacuum filtration. In this study 70% of sperm cells in the mixed cell sample penetrated the filter into the collection tube. This provides a larger sperm yield from that of preferential lysis. However, 1.0-2.0% of intact epithelial cells also penetrated the filter into the collected sperm, and epithelial cell DNA released from lysed cells contaminated the filtrate. The investigator then performed this separation on a 1:1 mixture followed by genotyping at the D1S80 locus with PAGE visualized by silver staining. Although no epithelial carryover was detected in the genotype, this outcome does not compare either to the level of sensitivity in current STR capillary electrophoresis analysis or the ability PCR multiplexing has in detecting a DNA mixture. This filtration method may be more suitable for samples containing large amounts of sperm with only trace contamination by epithelial cells.

Efforts are currently underway to develop a new cell separation technique using magnetic beads coated with antibodies [Herr, 2004]. This approach requires the identification and isolation of specific monoclonal antibodies that target the surface of the human sperm cell. A second antibody conjugated to a magnetic bead specifically binds to the first antibody and is then used to entrap the sperm cells and separate them from unwanted cells in a mixture. One of the challenges with any antibody-based method is the stability of cell surface antigens in environmentally compromised forensic samples. The magnetic bead technology itself is a proven technology and the forensic community awaits reports on the outcome of this research.

Y-chromosome STR analysis takes a non-separation approach to identifying a male's DNA from a female's DNA in a forensic mixture [Kauser et al., 1997]. The

identification of the Y chromosome haplotype of a perpetrator can be determined in a mixed male/female specimen in which the female cells are in overwhelming quantity [Prinz el al., 1997] while maintaining the high level of sensitivity seen in autosomal STR analysis. In addition, Y-STR analysis can clarify the numbers of semen contributors when multiple males are involved and is useful in rare incidences of azoospermic perpetrators. Y-STR analysis does not discriminate sexual assault mixtures involving the same sex, such as sodomy of a male and does not specifically target sperm cells. Therefore, if multiple assailants are involved in a mixture, determining the origin of the genotype to the semen deposited or cell type can be unclear. Cell source attribution can be important in defining the type of sexual assault crime committed in court.

The Y-chromosome lacks recombination from father to son and the statistical product rule cannot be applied as many markers are required to achieve a practical degree of discrimination [Butler, 2001]. However, even with a multiplex assay, Y-chromosome analysis does not reach the degree of statistical power that the 13 core STR loci provide, and it has yet to be incorporated into the FBI's Combined DNA Index System (CODIS). Furthermore, when a Y-STR database is formed, the State and Federal costs of Y-typing archived and newly convicted offender blood samples in addition to the 13 core loci will have to be addressed.

The methods described above have weaknesses in efficient cell separation, yield, time effectiveness, cost, ability to work with minute amounts of starting material, identifying genotype to a specific cell type, and ability to discriminate multiple perpetrators. This research project proposed Laser Microdissection as a new sample separation method to address some of these weaknesses. Laser microdissection (LMD)

technology has been increasingly used in biomedical research applications to harvest selected cells from histological sections of complex tissues [Emmert- Buck et al., 1996; Simone et al., 1998; Luizzi et al., 2001, Rook et al., 2004]. LMD has several potential advantages for forensic science over previous separation methods. It requires low-manipulation of sample and works by direct microscopic visualization, making it suitable for single-cell analysis. This resolution makes LMD ideal for forensic samples of minute quantities. LMD directly separates and collects the target cells without contamination from a mixed cell population. This separation can be verified visually by the post-dissection image of the slide. Also, the post-collection inspection mode in the software allows the user to microscopically inspect the contents of the collection tube. Thus LMD addresses the question about cell source type of the genetic profile obtained.

Laser microdissection technology was first introduced as "laser capture microdissection" or LCM (Arcturus). Recently LCM was reported to improve recovery of DNA from sperm on microscope slides [Elliott et al., 2003]. This technology involves the use of a laser to microscopically melt a thermoplastic film onto a target cell embedding and lifting the cell from the slide. Although this technology allows the capture of an enriched sperm fraction, female carryover is relatively common. This problem can be due to female DNA from lysed cells adhering to the sperm but it may also be due to the non-specific attachment of surrounding foreign cells to the plastic membrane. Despite contamination of female DNA in the male fraction LCM performs significantly better than the preferential lysis method in its ability to separate sperm from vaginal epithelial cells and to detect STR alleles from even a few sperm [Elliott et al., 2003].

Since the initial use of LCM, laser microdissection (LMD) engineering has evolved and other instruments developed. In this study the third generation Leica AS LMD instrument (Leica Microsystems) was evaluated and a method developed to separate pure populations of sperm cells from semen/epithelial cell mixtures compatible with forensic DNA analysis. The aim of this research was to develop a method to simultaneously identify and separate a pure population of sperm cells from an epithelial cell mixture applicable to forensic human identification testing.

Development of laser microdissection could assist forensic scientists in analyzing sexual assault mixtures that previously could not be effectively separated using conventional means. LMD could also be the tool of choice for collection of minute numbers of sperm in mixtures for Low Copy Number Analysis. With proper development of software and integration of robotics, LMD could become more automated and has the potential of processing at a higher throughput for backlog reduction.

Objectives

In our NIJ grant proposal, we proposed to use Laser Microdissection technology for the separation and recovery of sperm cells in sperm/epithelial cell mixtures. In particular we were interested in developing practical LMD methods for the simultaneous identification and separation of spermatozoa from sexual assault evidence mixtures for standard and low copy number STR analysis. Our three basic goals were 1) separation of cellular mixtures for standard STR analysis; 2) low copy number analysis with LMD and 3) compare and evaluate LMD and current separation methods. Achieving each of these

goals would advance forensic DNA analysis. For example, we anticipated that this LMD method would be especially useful tool for recovering sperm cells from a high density of epithelial cells in a mixture. Such a method would allow the analyst to recover a pure population of sperm cells without cross contamination from the epithelial cell fraction, thus highly useful for STR genotyping of the sperm donor. In addition, we expected that the LMD method would be particularly helpful for collection of just a few sperm for low copy number analysis, allowing the STR analysis of evidence samples that would typically be too minute for sperm donor genotyping using current mixture separation methods. Using such a newly developed tool, we projected an increase in success rate of DNA analysis in sexual assault cases. As described in our proposal our objectives were to develop a forensic LMD method for cell separation and also make the method available to the forensic community through presentations and publications.

Progress Toward Meeting Objectives

As we will describe in the remainder of this final report, we were successful in meeting the majority of our detailed objectives. We have developed LMD separation methods for the collection of sperm for both standard and low copy number STR analysis and contributed substantially to the SWGDAM guideline validation process (SWGDAM 2000). Much of the developmental work surrounding this research has been presented at forensic meeting as oral presentations (Sanders et al., 2004,2005,2006), one manuscript has been published (Sanders et al., 2006), and a second manuscript is currently in progress.

Throughout the development of the aforementioned LMD methods, we gained experience in the preparation, extraction and PCR amplification of LMD samples, and we began to identify critical issues for obtaining successful cell separation for STR analysis. We also received feedback following the various presentations of our data that assisted us in modifying details of our objectives and experiments to better address the issues faced by forensic scientists. In particular, we found that many of the histological stains tested had a larger harmful impact on downstream analysis than expected. We therefore devoted more time and resources in expanding the extent of the histology study to identify an appropriate staining method for simultaneous identification and dissection by LMD. Due to the impact chemical stains had on our samples we eliminated experiments that were to address the potential of LMD in removing exogenous inhibitors as success of this goal appeared unlikely. We also eliminated experiments regarding separation of sperm from blood mixtures addressing feedback from the forensic community that these types of mixtures would likely behave similarly to the sperm\epithelial cell mixtures and determined that the resources for this project would be best spent addressing other questions. Finally, we added the examination of genuine sexual assault case samples to test if they responded to LMD separation in a manner similar to the controlled mixed-cell specimens.

A Laser Microdissection Separation Method for Spermatozoa from Epithelial Cells for Standard STR Analysis

Introduction

Semen typically is the component of interest in sexual assault evidence involving a male assailant. Depending on the nature of the crime the victim's own cells are commonly commingled with spermatozoa, as there is an abundance of epithelial cells lining the vaginal, rectal, and oral cavities. In this study, LMD was evaluated and a method developed to separate pure populations of sperm cells from semen/epithelial cell mixtures suitable for DNA analysis. With any new technology introduced into the legal arena, validation studies are critical but also time consuming. We therefore found it important to incorporate an LMD protocol with commonly used upstream and downstream methods used in forensic biology laboratories, which we refer to as "standard" analysis in this report. By avoiding manipulating these well-established DNA methods, the LMD method described can be better implemented into practical use. A series of three experiments were performed to assess LMD technology. First, the effect of various histological stains on downstream analysis from LMD stained cells was examined. Second, DNA isolation methods were compared when applied to LMD collected cells. Finally, the separation capability of LMD to recover sperm cells from epithelial cells in a stained mixed specimen was determined.

Materials and Methods

Sample Collection

Liquid semen samples were obtained from stock internal laboratory standards stored at -20ºC. Working solutions of the semen samples were prepared to a 1:10 dilution in sterile water for all samples. Buccal swabs were obtained from female subjects by sterile cotton swabs, dried, and then stored at -20ºC. Each swab was agitated in sterile water and the epithelial cell pellet recovered in a 50µl working solution. All procedures involving human subjects were in accordance with the Rosalind Franklin University Institutional Review Board.

Samples/LMD Slide Preparation

Mixtures were prepared by combining 25µl of the epithelial cell pellet working solution with 10µl of the 1:10 semen working solution. Two microliters of both the mixtures and single source working solutions were smeared over a 7mm diameter circle on a PEN slide (Leica Microsystems, Brannockburn, IL) and dried at room temperature. The PEN slide is a glass microscope slide covered with a 2µm thick polyethylene napthalate (PEN) plastic membrane, which is adhered near the edges of the slide.

Histological Staining

Sterile filtered solutions of hematoxylin/eosin (H&E), nuclear fast red (NFR), nuclear fast red/picroindigocarmine (CTS, also known as “Christmas tree stain”), methyl green (MG), Wright’s stain (WRT) and acridine orange (AO) were used for staining of

cells. The durations for which slide smears were exposed to chemical stains in the histology comparison study were as follows: H&E - Mayer's hematoxylin for 5 minutes then eosin for 5 minutes; NFR - nuclear fast red for 5 minutes; CTS - nuclear fast red for 5 minutes then picroindigocarmine for 30 seconds; WRT - Wright's stain for 5 minutes; AO - acridine orange for 4 minutes; and MG - methyl green for 5 minutes. Unstained control smears were rinsed with 95% ethanol for 5-10 seconds. A modified protocol of hematoxylin/eosin staining (H&E Modified) was performed where indicated in which exposure times to chemical stains were reduced to the following: Modified H&E - Mayer's hematoxylin for 1 minute then eosin for 10 seconds. In addition, the LIVE/DEAD® Sperm Viability Kit from Molecular Probes (Invitrogen Corp., Carlsbad, CA) containing SYBR 14 and propidium iodide (PI) was tested under manufacturer recommended conditions altering the PI to a 50-fold dilution. All slides were vacuum-desiccated and stored at -20ºC. Desiccation was repeated at room temperature immediately before laser microdissection.

Laser Microdissection

The Leica AS LMD instrument (Leica Microsystems, Bannockburn, IL) is a computer controlled, motorized, upright laboratory microscope integrated with a 337nm UV laser. The cells of interest are visualized and marked through the computer software. Then the pulsed laser beam is directed through the objective lens passing through the inverted glass microscope slide to the plastic PEN film on which the sample resides. Laser ablation occurs around the cell(s) of interest and the material is collected by gravity into the cap of a PCR tube below the stage. The technique is illustrated in figure 1.

In facilitating accurate cell counting for this study, laser microdissection collection was limited such that clusters of sperm cells were avoided. This was done by limiting single software tracings to one to ten cells at any one time. Slides were vacuum desiccated and brought to room temperature immediately before LMD. The LMD parameters used at the 40x objective are listed in Table 1.

An analog hand counter was used while sperm and epithelial cells were dissected by LMD from the prepared smears. Cells were automatically collected into the caps of 0.2ml thin walled PCR tubes containing 20µl of the appropriate collection buffer for each DNA isolation method described below. After collection cells were centrifuged down from the cap for 10 seconds.

DNA Isolation

Qiagen QIAamp - LMD cells collected in TE buffer (10mM Tris-HCl, 0.1mM EDTA, pH8) were extracted using the QIAamp® DNA Micro Kit (Qiagen, Valencia, CA). The DNA isolation was performed according to the manufacturer's recommendations for microdissected samples with the addition of dithiothreitol (DTT) to a final concentration of 30mM in the lysis step. Final elution volume ranged from 20-25µl.

MicroLYSIS - LMD cells collected in MicroLysis® reagent (Microzone Ltd., West Sussex, UK) were extracted using 20µl of reagent with the addition of DTT (30mM) and incubated in the thermal cycler according to the manufacturer's

recommendations as follows: 65ºC for 5 minutes, 96ºC for 2 minutes, 65ºC for 4 minutes, 96ºC for 1 minute, 65ºC for 1minute, 96ºC for 30 seconds, 20ºC hold. LMD sample collection, lysis and PCR were all performed in the same 0.2ml thin-walled tube.

Lyse-N-Go - LMD cells collected in Lyse-N-Go™ reagent (Pierce Chemical Co., Rockford, IL) were extracted using 20µl of reagent with the addition of DTT (30mM) and incubated in the thermal cycler according to the manufacturer's recommendations as follows: 65ºC for 30 seconds, 8ºC for 30 seconds, 65ºC for 90 seconds, 97ºC for 180 seconds, 8ºC for 60 seconds, 65ºC for 180 seconds, 97ºC for 60 seconds, 65ºC for 60 seconds, 80ºC for 5 minutes. LMD sample collection, lysis and PCR were all performed in the same 0.2ml thin-walled tube.

PCR Conditions

DNA Amplification was performed using the AmpF*l*STR® Profiler Plus Kit™ (Applied Biosystems, Foster City, CA) for 9 STR loci (D351358, vWA, FGA, D8S1179, D21S11, D18S51, D5S818, D13S317, D7S820) plus amelogenin using a Bio-Rad iCycler to carry out the PCR. Standard PCR was performed according to manufacturer recommendations as follows: 21µl AmpFlSTR PCR Reaction Mix, 1µl AmpliTaq Gold® DNA Polymerase, 11µl AmpFlSTR Profiler Plus Primer Set, 20µl sample DNA; Thermal cycling conditions - Incubate 95ºC for 11 minutes (polymerase activation); 94ºC for 1 minute (denaturation), 59ºC for 1 minute (annealing), 72ºC for 1 minute (extension) for 28 cycles; then 60ºC for 45 minutes (final extension). In addition, extended cycles were used with PCR conditions as follows: 25µl of PCR product amplified under the standard

conditions were removed and added to a new tube with 0.25µl of AmpliTaq Gold® DNA Polymerase, then PCR performed for six additional cycles as above.

Electrophoresis Conditions

One and a half microliters of each PCR product was denatured in 24µl of HI-DI formamide with 1µl of ROX 500 size standard (Applied Biosystems, Foster City, CA). Electrophoresis and data collection were performed on a ABI Prism 310 Genetic Analyzer (Applied Biosystems, Foster City, CA) using a 5 second injection time for the histology study and an ABI Prism 3100 Genetic Analyzer using a 11 second injection time for the DNA isolation and mixture studies.

Data Analysis

GeneScan 3.1.2 and Genotyper 2.5.2 (Applied Biosystems, Foster City, CA) software was used to analyze the electrophoresis data. Baseline correction, matrix correction and light smoothing were applied to all samples. The PCR amplification of human DNA using the Profiler Plus™ kit is such that a florescent dye is incorporated into each amplicon through a 5'-end labeled oligonucleotide primer, therefore, the fluorescent signal detected is a measure of quantity of the amplified target. Sample peak heights, in relative florescent units (RFU), of all true alleles were used for quantitative analysis and heterozygous peak ratio calculations. The minimum peak height threshold was set at 50 RFU to allow for detection of all peaks clearly above background. Data compilations were performed using Microsoft Excel 2001 and GraphPad Prism 4.0 including mean, standard error, unpaired t-test, and ANOVA analysis with the Bonferroni post hoc test.

Studies

LMD Histology Comparison - The histology study involved two separate comparison groups. Group 1 was initially designed to test a larger panel of stains to quickly identify an appropriate stain to use in subsequent studies. To improve upon the STR results obtained from the group 1 study, a second comparison was performed. Group 2 involved the comparison of modified group 1 staining protocols and the addition of testing of the LIVE/DEAD® Sperm Viability Kit.

The group 1 comparison involved samples from 6 donors (3 semen and 3 oral swabs). Six slides were prepared for each single source donor specimen and stained with H&E, CTS, MG, WRT, and AO including an unstained control as described in the histology methods. Cell identification was performed at a magnification under the 40x and 63x objectives using brightfield and florescence microscopy on the Leica AS LMD microscope. Scores were assigned to describe the stain's ability to facilitate cell identification as follows: double minus (- -) = cannot ID or highly challenging; minus = poor; plus/minus (+/-) = satisfactory; plus (+) = good; double plus (+ +) = excellent. Collections of 300 sperm cells and 150 epithelial cells were recovered by LMD representing equivalent amounts of starting DNA material from the haploid sperm and diploid epithelial cells. Cells were isolated using the Qiagen QIAamp® DNA isolation method followed by STR analysis using standard 28 cycle PCR conditions with 20µl of undiluted DNA extract from all samples. RFU values were tabulated for each sample at all loci and compared to values of the unstained specimen to determine relative PCR product yields.

The group 2 comparison involved samples from 10 donors (5 semen and 5 oral swabs). Four slides were prepared for each single source donor specimen and stained with Modified H&E, NFR (CTS without PI), and SYBR14/ PI including an unstained control as described in the histology methods. Cell identification was performed at a magnification under the 40x and 63x objectives using brightfield and florescence microscopy on the Leica AS LMD microscope. Collections of 150 and 300 cells from both sperm and epithelial smears were recovered by LMD. Cells were isolated using the Lyse-N-Go™ DNA isolation method followed by STR analysis using standard 28 cycle PCR conditions. RFU values were tabulated for each sample at all loci and compared to values of the unstained specimen to determine relative PCR product yields.

LMD DNA Isolation Comparison - Samples from 10 donors (5 semen and 5 oral swabs) were examined. Collections of 300 sperm cells and 150 epithelial cells stained with H&E Modified were collected by LMD in triplicate to compare Qiagen QIAamp®, microLYSIS® and Lyse-N-Go™ DNA isolation methods. All samples were processed under standard PCR conditions. RFU values were tabulated for each sample at all loci to determine relative PCR product yields.

LMD Mixture Study - Five mixed cell samples from 10 donors (5 semen and 5 oral swabs) were examined. Collections of 300, 150 and 75 sperm cells stained with H&E Modified were separated by LMD from the mixtures. A serial dilution of a human DNA standard was included in the analysis, amplifying 2ng, 1ng, 0.5ng, 0.25ng and

0.125ng of DNA to compare to LMD collected cell samples. All samples were processed using Lyse-N-Go™ DNA isolation applying both standard and extended cycles PCR.

Results

Histology Comparison

Group 1 - To ascertain an appropriate histological stain for LMD recovery several common stains were tested for their utility in sperm and epithelial cell identification and their effects on downstream DNA analysis. Identification scores were assigned to cells microscopically examined from PEN slides without a coverslip (Table 2). Unstained specimens could be identified under brightfield conditions although the process at times was slow and laborious when sperm tails were detached. Both hematoxylin/eosin and Christmas tree stain readily provided morphological discrimination of spermatozoa and epithelial cells. Both Wright's stain and methyl green staining resulted in poor visualization of sperm cells making identification difficult. The penetration of methyl green was difficult to control and the Wright's staining method appeared to cause some deformation either in the epithelial cells or the PEN membrane hindering identification. Acridine orange performed well for identification of sperm, although, it appeared that differentiation amongst a concentrated field of epithelial cells might be challenging as the larger epithelial cells brightly fluoresced, potentially masking hidden sperm cells. Because measures were based upon a single evaluator, statistical analysis was not applied.

STR data from hematoxylin/eosin, Christmas tree stain, and acridine orange and unstained cells were compared. Figure 2 illustrates capillary electrophoresis data in the blue spectra showing a decrease of RFU peak height exhibited by stained epithelial cells. Total RFU values of all Profiler Plus loci from the stained sperm and epithelial cells were compared to that of the unstained control to determine relative percent PCR yield. Combined data from sperm and epithelial cells were analyzed by ANOVA followed by Bonferroni post hoc test. One semen donor sample was excluded from statistical analysis due to the amplification failure of five loci from an unstained control. Stained specimens showed a significant decrease in total RFU values compared to unstained specimens. H&E samples exhibited RFU values 62.4% $\pm$6.6% of that observed by the unstained control ($p < 0.01$). CTS samples exhibited RFU values 42.6% $\pm$ 5.5% of that observed by the unstained control ($p < 0.001$). Cells stained with AO produced no amplified product in all samples tested.

Group 2 - To improve STR results observed in the group 1 experiments an additional comparison was performed. Incubation times for H&E staining was reduced and nuclear fast red was used alone, omitting the picroindigocarmine from the CTS staining protocol. STR data from modified H&E, NFR, and unstained cells were compared. Figure 3 shows total RFU values of all Profiler Plus loci from the stained and unstained sperm and epithelial cells. Total RFU values did not significantly differ between the H&E or NFR stained specimens and the unstained controls ($p > 0.05$, ANOVA Boneferroni post hoc test). Cells stained with SYBR14/ PI produced no amplified product in all samples tested.

DNA Isolation Comparison

Three isolation methods were compared to evaluate their effectiveness in DNA extraction from stained cells and to develop a method enabling successful STR analysis of LMD samples. Figure 4 shows mean values of the number of loci detected from samples processed with three different isolation methods. MicroLYSIS® performed poorly for both sperm and epithelial cell samples with a high degree of allelic drop-out. Both Lyse-N-Go™ and QIAamp® methods successfully isolated sperm DNA ("300 cell" count) such that all loci were detected in 100% of the samples, however, results from epithelial cell extractions ("150 cell" count) varied. On average 74$\pm$6.8% of the female donors' loci were detected using Lyse-N-Go™ on epithelial cells, while 90$\pm$5.4% of loci were detected using the QIAamp® method. However, within this study population the difference could not be deemed statistically significant ($p > 0.05$, ANOVA Bonferroni post hoc test).

RFU (signal intensity) is a measure of PCR product quantity. Therefore, RFU values from Lyse-N-Go™ and QIAamp® samples were compared to evaluate amount of PCR product produced. Figure 5 summarizes the total fluorescence signal detected in total RFUs (the sum of the peak heights at all loci) for each LMD sample comparing Lyse-N-Go™ and QIAamp® methods. QIAamp® extractions produced RFU values approximately 75% higher than the Lyse-N-Go™ method, a significant increase ($p < 0.05$, paired t-test), when used to extract epithelial cells. When applied to sperm cells the Lyse-N-Go™ method resulted in higher observed RFU values in 5 out of the 6 samples compared to the QIAamp® method. However, average RFU values did not exhibit a

statistically significant difference within this sperm sample population ($p > 0.05$, paired t-test).

Mixture Separation Study

To establish the separation capability of LMD, sperm cells from semen and female oral epithelial cell mixtures were recovered followed by DNA analysis. STR plots of sperm cells recovered from semen/buccal mixtures are illustrated in figure 6. In all samples tested, the semen donors' genotypes were detected with the absence of any alleles known to originate from the female buccal cell donors. Under standard PCR conditions (28 cycles) all samples containing 300 LMD sperm displayed all 10 loci of the sperm donors. Samples containing 150 sperm exhibited on average 96$\pm$3% of the male donors' alleles and samples containing 75 sperm cells displayed on average 72$\pm$12% of the male donors' alleles. The number of alleles detected above threshold ($\geq$50 RFU) is tabulated for each specimen in Table 3.

Using extended cycles (6 additional cycles) PCR, 100% of samples containing 75 and 150 sperm cells exhibited all of the sperm donor alleles. Non-overlapping female alleles were not detected in any samples using a total of 34 PCR cycles demonstrating the collection of a pure population of sperm cells without female DNA contamination as illustrated in figure 7. Data from extended cycles PCR containing 300 sperm were not included in this report due to the preponderance of peak heights above the linear range of the instrument and an abundance of PCR artifacts typical of increased PCR cycles such as increased stutter and minus A nucleotide products.

The relative quantities of PCR product were examined from the three LMD

collection amounts of sperm cells. Figure 8 summarizes the total fluorescence signal detected at each locus for the three collection amounts using standard and extended cycles PCR. In general the total RFUs detected increased with an increase in the number of LMD collected sperm cells. Assuming one human haploid cell contains 3.3pg of genomic DNA, the examination of 75, 150, and 300 sperm cell amounts contains approximately 0.25, 0.5 and 1.0ng of DNA respectively prior to DNA isolation. Signal intensity was compared from the experimental samples with a dilution series of the AmpF*l*STR® DNA positive control under standard PCR conditions. Figure 9 shows a plot with regression line of the positive DNA control analyzed from 0.125 to 2ng. As expected the positive control showed a linear relationship between RFU value and quantity of DNA (r^2= 0.9961). Mean values of the LMD samples plotted on the same graph maintained a linear relationship (r^2= 0.9179) with RFU values 2.5 - 3.7 times less than the positive control values. This reduction likely reflects DNA isolation inefficiency and is explored further in the yield evaluation study in the last section of this report. Peak height ratio, which is defined as the height of the lower peak divided by the height of the higher peak - expressed as a percentage, was calculated at heterozygote loci. Samples that displayed only one allele at a locus where the donor was heterozygous were excluded from the calculations. Peak height ratios are displayed in figure 10. Mean peak height ratios over all loci under standard conditions were 76.3$\pm$3.3% for "75 sperm", 81.1$\pm$1.3% for "150 sperm and 82.0$\pm$1.4% for "300 sperm" samples. Mean peak height ratios for extended cycles analysis were 67.0% $\pm$ 4.2% for "75 sperm" and 85.2$\pm$2.1% for "150 sperm" samples.

Discussion

The results of this study demonstrate through STR genotyping that laser microdissection of mixed cell populations achieve pure separation of sperm with no DNA contamination from exogenous buccal epithelial cells. The considerations for using laser microdissection are discussed below.

LMD Technical Discussion

Several laser microdissection systems were considered for this project. Three systems were further investigated for their suitability in this research. Arcturus Pixcell developed the first laser microdissection system, which was patented as "laser capture microdissection" (LCM). The LCM technology uses an IR laser to microscopically melt a thermoplastic film onto the cells of interest. This process embeds the cells in plastic followed by pulling the cells by force from the slide. Some concerns arose regarding the nature of this type of collection. The plastic film is held in a cap holder than comes into physical contact with a large area of the slide. This instrument was initially designed for tissue cryosections where the integrity of the tissue section can withstand the contact. However, it was suspected that with a slide smear this could result in a simple contact transfer of unwanted cells from the slide to the surface of the plastic thus contaminating the cap. With the sensitivity of PCR and the increasing popularity of Low Copy Number Analysis contact transfer can now be detected [Ladd et al., 1999]. Similarly, since the method does not employ cutting there were concerns that cells adjacent to or overlapping the target cell - such as a sperm head on the non-nuclear body of an epithelial cell - may lift the whole contaminating cell onto the cap. Finally, the operation of the instrument is

performed in real-time somewhat like a video game where the operator controls a joystick type device directing the stage and firing the laser to collect cells. This appeared to be labor intensive and allowed little room for operator error. The Acturus system did have one advantage in that specimens could be recovered from a standard glass microscope slide. This could be useful when faced with archival samples not specifically prepared for laser microdissection. However, old mounted specimens could still prove challenging as specimens on aged slides have a propensity to be more permanently adhered to the glass slide.

The second instrument considered was the P.A.L.M MicroBeam laser microdissection and pressure catapulting (LMPC). This system has the improvement of non-contact collection. It uses a laser to cut the specimen, however, since the slide sits upright the target material must be catapulted up against gravity using a pressure pulse. According to the manufacturer it is "like a ball that is kicked into the goal". The "goal" is an inverted microtube cap. The sample is held onto the underside of the cap by either tension or an adhesive cap. We were uncomfortable with the engineering of this type of upward collection process in retaining all cells given it is working against gravity, however, we did not perform any tests on this instrument to support or disprove our concerns. Finally, the most basic of PALM systems started at a cost well above the other systems considered.

It was determined that the Leica Microsystems AS LMD would be best suited for this project’s application. As illustrated in figure 1, tracings are made through the computer software around the area of interest. The cutting technology, known as laser ablation, is based on a pulsed UV laser which cuts around the cell(s) of interest which

then drop into a tube below the stage. This technique avoids direct UV irradiation or mechanical contact, which offers the advantages of preventing contamination and secure recovery of intact DNA.

The Leica AS LMD is designed primarily to cut mounted tissue cryosections. Since cell smears were used in this study, some time was spent on determining the optimal sample preparation and cutting conditions for this study. The instrument has several available cutting parameters what can be manipulated as show in Table 1. The UV laser operates at 30Hz and up to 60 Hz in burst mode. The intensity of the laser can be adjusted using a "less" to "more" sliding scale. It was observed that determining the minimum necessary energy to cut the material in question was important because using excessive laser strength caused leakage of laser light to a nearby area of the slide. Shadows of the cutting pattern were occasionally observed about 100-200µm from the target cut when the laser was operated at maximum power. Although the intensity of the deflected light was not strong enough to cut through the material, there was obvious ablation damage to the surface. It was conceivable that this leakage from the UV laser could degrade or destroy nuclear material from nearby cells if not controlled.

The cutting speed parameter can be adjusted on a slow to fast sliding scale. By slowing down the laser speed, a deeper cut could be achieved and laser intensity reduced. The drawback although to reducing speed was an increase in collection time. The balance between laser intensity and speed were the two parameters that had the most dramatic effect on cutting performance. However, once the desired settings were determined for a particular type of specimen the specification could be saved and quickly restored by the computer software for future cutting sessions. This allowed the

instrument to be used for a variety of other applications such as pathology samples, hair roots or other tissue without re-optimizing for each different tissue type or user prior to each session.

Laser aperture can also be adjusted on a sliding scale. A narrow aperture gave more precision and control in excising specific cells without the ablation of adjacent cells while a wider aperture better facilitated the drop of the specimen into the collection tube. It was found however that the larger apertures were only necessary when large, odd-shaped, free-drawn cuts were performed. It is analogous to a jigsaw puzzle where an elaborate piece would be more difficult to punch out than the circular piece. In this study most of the specimens involved collection of only one or two cells no larger than a circle with a 30-micron diameter and throughout the experiments performed, a narrow aperture was found to be sufficient for dissection of these small pieces.

Toward the end of each cut, the instrument pauses at a short distance from completion of the closed loop. A larger and wider burst of laser power is then pulsed to push the cell(s) into the collection tube. Both the gap (small, medium, large) during the pause and the amount of increased laser intensity and aperture can be controlled. In general, longer bridge gaps allowed more controlled sample drops. The shorter the gap the more frequent the PEN membrane would prematurely peal away before the final burst potentially causing a hanging sample which we named "hanging chad" after the 2000 presidential election controversy. The sample would hang below the slide attached only by a small bridge. This premature movement could cause the focal plane to be offset from the remaining membrane to be cut. Because the effectiveness of the laser is in part dependent on the microscope objective's focus on the specimen, the final burst

occasionally did not complete the final cut when hanging chads occurred. This problem may be prevented in two ways. 1) Longer gap settings 2) Wider more intense final laser bursts. However, once a hanging chad occurs it was found that the best method effective in completing the cut and collection was to refocus and retarget the bridge using the line cut feature. Optimal LMD parameters were determined for sperm and epithelial cells as described in the methods section. In general, slower more intense laser settings were required to collect epithelial cells.

The 4x, 10x and 20x objectives were insufficient to identify the small sperm cells of 5µm. The 63x objective was helpful in identifying or verifying sperm morphology and could also be used for dissection of spermatozoa. Although, the 63x objective created a smaller field of view reducing the potential number of sperm cells that could be cut at any one time thereby slowing the recovery process. The 40x objective performed the best for simultaneous identification and dissection of both sperm and epithelial cells.

Images of sperm/epithelial cell mixtures cut by LMD are show in Figure 1. Circles were found to be the most effective in targeting sperm cells. By using the circle and reuse tool option, which replicates the previously used cutting shape and size, it was possible to easily target and excise more sperm cells. Moreover, epithelial cells could also be recovered in a separate collection tube either simultaneously or after sperm collection. Changing to more aggressive cutting parameters (Table 1) could collect sperm cells overlapping with epithelial cells. If a sperm cell resided very close to an epithelial cell nucleus ablation of the nucleus could destroy epithelial cell DNA before collection of the sperm cell to avoid contamination.

Sample preparation requires some unique challenges when performing laser microdissection. Two different types of slides were evaluated: polyethylene terephthalate (PET) and the polyethylene napthalate (PEN) slides. Leica Microsystems first developed the PEN slide followed by the production of PET slides in response for the need for an inert material for mass spectrophotometry applications. No cover slip is used in laser microdissection, therefore, the quality of the image is reduced compared to traditional microscopy. One of the characteristics explored in this study was the plastic clarity of the PET over the PEN slides. The PEN slides contain what appear to be minute pores in the membrane reducing the optical clarity of cell smears. According to the distributor, PEN material could be manufactured without the production of pores and at the time this report was actively working with the manufacturer to create such a product. However, PET film is already nearly absent of any pores. Several samples were prepared and examined on the PET slides but we found some distinct disadvantages over the PEN slides. The PET membrane is stretched over a metal frame such that it hangs open with no contact against a solid surface. Preparing smears onto this fragile membrane was difficult, as even if the smear was gently applied and supported against a solid surface, deformities frequently occurred on the membrane. This exasperated the problem of continuous focal adjustment from field to field on the microscope. The PET membrane was also more difficult to cut requiring increased laser intensity and sometimes repeat cuts. Samples that contain very few sperm heads, which are difficult to identify, could benefit from using PET slides, however, it was determined that the drawbacks outweighed the optical advantages for routine analysis.

Histological techniques to improve the optical quality of sectioned tissue specimens are worth exploring but were beyond the scope of this project. Clearing agents have been used to de-fat tissues and used routinely for laser microdissection followed by nucleic acid analysis [Ehrig et al., 2001]. However this would involve more toxic chemicals and its effects on forensic STR analysis would have to be investigated. Also, a recent development using a thin aqueous mounting solution with an adhesive gum base has been reported to greatly improve tissue morphology for laser microdissection. No detrimental effects were detected on DNA isolation and efficiency of PCR amplification including only a minimal reduction of LMD cutting efficiency [van Dijk et al., 2003]. Another practice of simply dehydrating the slides by rinsing through a gradient of low to high percentage ethanols is a technique that would not be expected to affect any downstream analysis. This could improve clarity and would also better dehydrate the specimen, an important component described in more detail below.

The cell concentration of the slide smear is also a consideration. In preliminary studies 2-5µl of the cell pellet were tested over quarter inch diameter circles on the slide. In general the smaller aliquot smeared over the spot area allowed a wider distribution of cells reducing the occurrence of sperm cells overlapping with epithelial cells. Most of these initial samples tested did not contain high densities of epithelial cells, which are frequently encountered in postcoital vaginal swabs. It was anticipated that with increasing numbers of epithelial cells the smears may have to be adjusted such that a larger area is utilized on the slide or the cell pellet is diluted before slide preparation.

Forensic laboratories are familiar with the need to control a facility's environmental conditions within the laboratory to maintain consistent operation of

sensitive analytical equipment, and integrity of evidence samples. For example some DNA sequencers are sensitive to room temperature changes effecting electrophoresis mobility. In addition, excessive moisture and/or heat can compromise biological specimens if not stored properly. Control of environmental conditions was found to be critical for successful laser microdissection. A slide specimen had to be well dehydrated before dissection. Water will dissipate the laser energy resulting in poor ablation. If moisture remained on the slide after a cut has been made on the plastic membrane the water could migrate between the glass slide and the membrane, making cutting nearly impossible. It was important when removing slides from the freezer to immediately vacuum desiccate to prevent the accumulation of condensation on the surface. However, in contrast excessively dry air conditions created static electricity, which sometimes caused a cut piece of PEN film to be attracted to the underside of the slide instead of dropping into the PCR tube. This problem was discovered as changes in cutting performance were observed through the cold, dry winters and hot, humid summers. The humidity was controlled inexpensively by using a simple room dehumidifier in the summer and a humidifier in the winter to reach the ideal humidity of 35-45 percent for LMD cutting. This avoided static electricity while preventing the absorption of water by the sample during the collection period. By controlling the humidity there was also a reduction in the effect of another dilemma with LMD collection, evaporation of buffer from the collection cap.

The collection cap has an effective working volume of 20-30µl using the 0.2ml PCR tube option (0.5ml tube option available). The brightfield illumination originates from below the stage and travels through the cap to the slide above. The heat from this

light and the open exposure of the buffer to the environment can quickly cause evaporation of this small amount of buffer from the collection cap. Steps were initiated to address this problem. First, a filter was used to reduce heat exposure to the cap, and the bulb intensity was dimmed to a lower level. The digital video camera was able to compensate for the lower light level. Second, the collection cap can be put into a standby position where light is not transmitted through the cap and moved into position just before each cutting was initiated. The second method although effective in reducing the heat on the cap was time consuming as each time a new field of view was changed the tube had to be directed out of position then back into position. However, this bottleneck is expected to improve with pending software improvements better automating the process. In the meantime the first method was effective in minimizing volume loss. In this study an acceptable ~ 5µl volume loss during a 45-minute cutting session was experienced. Volume could be compensated by the addition of 5µl of either buffer or water to the collection cap prior to LMD. Given the evaporation loss there should be minimal dilution effect.

Since the inception of this study Leica Microsystems has released a new LMD instrument and improved software. Based on some of preliminary demonstrations of this instrument there appears to be several improvements that address concerns we may have had with the LMD instrument used in our study. The new LMD6000 has a solid-state laser at 355nm allowing more laser power delivered through the system. This has increased the cutting speed, which according to the manufacturer is 5 times faster. This improved speed appears to "drive" the samples down at the end of each cut avoiding the "hanging chads" we had described previously. Other improvements include more precise

scanning stage, larger slide and tube capacity, and more automated systems including auto-detect software, all which would likely increase throughput of sample collection.

Histology Study

The separation and recovery of sperm cells by laser microdissection for DNA analysis differs from the preferential lysis method in that LMD is best performed when the material is stained for a more accurate and efficient microscopic identification of the cells of interest. It is important that histological dyes chosen do not interfere with downstream analysis of the sample DNA material. PCR inhibition of genomic DNA by dyes and fixatives has been observed with gross, stained tissue samples (Murase et al., 2000; Serth el al., 2000). However, negative effects on DNA analysis from histological dyes can be reduced when the tissue is recovered using laser microdissection instead of manually dissecting tissue (Ehrig et al., 2001). This contrast is most likely attributable to the amount of tissue sampled as the cellular material collected by LMD is microscopically small and the instrument's ability to precisely excise the area of interest results in a low contamination of dye substances into further downstream analysis.

The objective of this part of the study was to identify dye chemistry with the least risk of degradative or inhibitory properties while still achieving good visual identification of the target cells. Several stains were chosen to investigate their effect on downstream analysis of LMD collected material. Nuclear fast red/picroindicocarmine, also known as the Christmas tree stain, is universally used to differentiate sperm cells from epithelial cells in stain identification of sexual assault evidence (Oppitz 1969). Hematoxylin/eosin is conventionally used as a nuclear stain in pathology laboratories and has been

successfully used to recover LMD tissue for nucleic acid analysis (Ehrig et al., 2001). H&E is also a popular choice for differentiation of sperm cells from epithelial cells in European forensic laboratories. Methyl green is a one-component nuclear dye believed to bind to the negatively charged DNA in nuclei showing no adverse effect on laser microdissected tissue by producing consistent amplification from manually dissected tissue (Murase et al., 2000). Wright's stain (azure blue/eosin) is a commonly used stain for blood smears (Walker et al., 1990). Acridine orange is a fluorescent stain used to visualize sperm from vaginal swabs particularly from samples with dense epithelial cell populations (Mercurio et al., 1997). The LIVE/DEAD® Sperm Viability Kit is a fluorescence based assay for determining the viability and fertilizing potential of sperm consisting of a membrane-permanent nucleic acid dye, SYBR® 14 dye, and a dead-cell stain, propidium iodine (PI) (Molecular Probes, 2001).

The overall performance of each stain was determined by considering both cell discrimination ability and genotyping results. Of the histological stains evaluated in the Group 1 study, H&E performed best. It readily provided morphological discrimination of spermatozoa and epithelial cells which is consistent with findings reported by Allery (Allery et al., 2001). The use of H&E however resulted in lower RFU values compared to unstained specimens. This supports reports indicating that hematoxylin produces less PCR product than unstained controls in laser microdissected tissue sections (Murase et al., 2000; Ehrig el al., 2001). Although the mechanism responsible for the reduced yield is not completely understood, hematoxylin-bound DNA seems resistant to complete digestion, which may make the DNA less available for enzymatic replication (Burton et al., 1998). In addition, while Eosin Y has shown no effect on PCR yield in laser

microdissected tissues (Murase et al., 2000), it is an acidic dye that could be responsible for DNA damage. Despite the observed reduction in PCR product, the use of H&E did not prevent the acquisition of sufficient PCR product for successful STR genotyping. In the group 2 study shortened exposure times of H&E staining were used as a simple tactic to reduce the uptake of dyes by the cells and lessen the negative effect of these chemicals in subsequent studies. This modification was successful as the results show that the modified H&E staining did not decrease the STR product yield.

Although the Christmas tree stain provided excellent morphological discrimination of spermatozoa and epithelial cells, its use produced significantly lower RFU values than H&E specimens ($p < 0.05$, paired t-test). This loss may be due to the picric acid component as highly acidic solutions will depurinate nucleic acids (Moore el al., 2002) damaging DNA. In addition, indigo carmine, used in the textile industry for dyeing denim, is a known inhibitor of PCR (Larkin et al., 1999) further causing low yields. Use of nuclear fast red stained paraffin-embedded tissues prior to laser microdissection has produced superior yield over other histological stains (Burton et al., 1998). Informal tests found NFR was sufficient for morphological identification of sperm and epithelial cells and was used alone in the group 2 study to improve yield. Like the modified H&E stain, NFR staining did not decrease the yield of STR products.

Acridine orange may have provided good visual identification of sperm but differentiation from epithelial cells became more difficult amongst high concentrations of epithelial cells and/or sperm without tails. It however proved to not be compatible with downstream analysis. AO intercalates with double stranded DNA and binds electrostatically to the phosphate backbone (Lerman et al., 1961), which may hinder

primer access to the template.

SYBR 14/PI staining from the LIVE/DEAD® Sperm Viability Kit provided good florescent identification of sperm and epithelial cells but was not compatible with downstream analysis. Early test samples of PI stained cells amplified and typed successfully for STR loci (data not shown), therefore, SYBR 14 is the likely cause the for the amplification failure.

DNA Isolation Comparison

Unique challenges were faced when developing and determining which DNA isolation method is appropriate for LMD cells. When confronted with molecular DNA analysis of histologically stained cells, a method that could remove Taq inhibitors from the sample would be advantageous. At the same time, conservation of DNA from the recovered cells is crucial. The time required to collect sperm cells is approximately 15-20 minutes per 100 sperm cells using the Leica system software version 4.1.3. , therefore, minimizing the necessary number of cells required reduces overall analysis time. In addition, a DNA isolation method that conserves the DNA and provides a concentrated extract such that the entire quantity can be used for PCR is important for the recovery of very minute evidence samples for subsequent low copy number (LCN) analysis.

Samples collected by LMD are held in a collection cap, which has a working volume of 20-30µl. The goal of this study was to incorporate and develop a DNA isolation method that can work in this small volume format and preferably in a single-tube format amenable to automation. Although Chelex is widely used in forensic casework (Walsh et al., 1991), preliminary studies of this project demonstrated that the

use of Chelex resin was a poor method for the extraction of DNA from LMD cells, as it was difficult to use in a low volume format, challenging to remove all the liquid from the resin beads, and resulted in little or no interpretable STR results (data not shown).

QIAamp® spin columns have been successfully used to isolate DNA from forensic casework (Greenspoon et al., 1998) and laser capture microdissetion samples (Martino et al., 2004). This method uses a column containing a silica-based membrane that binds nucleic acids. Through a series of washes and elution steps proteins and other contaminants, which can inhibit PCR and other downstream enzymatic reactions, are removed. This method provides a relatively pure DNA extract but requires sample transfers, washes and elution steps that may increase the chances of sample loss and potential cross contamination.

An alternative approach to DNA extraction is the use of one-step commercial buffers such as MicroLYSIS® reagent and Lyse-N-Go™ reagent, which are designed to lyse cells ready for PCR in one tube. These solutions allow the release of DNA through a series of heating and cooling causing the cells and their organelles to lyse open in addition to promoting inactivation of endogenous nucleases. LMD collection, lysis and PCR can all occur in a single tube requiring little manipulation resulting in conservation of the sample and prevention of sample-to-sample contamination.

The results of this study showed that the QIAamp® method performed best for the DNA analysis of stained LMD recovered epithelial cells by clearly producing higher RFU values than the Lyse-N-Go™ method. However, this was not the case when the cells isolated were sperm. Average RFU values did not differ between the Lyse-N-Go™ and QIAamp® methods when used for DNA isolation of sperm cells. This contradiction

may be explained by differences in amount of cellular material collected by LMD from the two different cell types.

The nucleus of a human cheek cell is approximately 5 microns in diameter, whereas a sperm cell head is approximately 5 microns by 3 microns. In this experiment, the nuclear material of the buccal cell was collected by recovering the whole epithelial cell body that is several times larger than the sperm cell. The amount of biological material including bound histological chemicals is therefore expected to be greater from the epithelial cells than the much smaller collected sperm heads. The QIAamp® kit is designed to remove proteins and possibly other contaminants that can inhibit PCR improving DNA yield from the epithelial cell samples. No such purification is done using the Lyse-N-Go™ method leaving effective contaminants in the PCR reaction. In contrast the sperm cell samples most likely contributed a smaller concentration of inhibitory histological dyes into the PCR reaction than the LMD collected epithelial cells. This may have allowed the sperm cell samples to benefit from the Lyse-N-Go™ method's ability to conserve sample.

The technique of diluting a DNA extract to reduce inhibitors and facilitate amplification, though with reduced sensitivity, is well documented (Wilson 1997). A similar approach of dissecting only the nuclei of the larger epithelial cells may reduce the contribution of inhibitory or degradative dyes into downstream analysis while maintaining the same concentration of DNA. In addition, it can be anticipated that as fewer amounts of cells are collected by LMD the concentration of inhibitors would decrease making collection of minute numbers of cells for Low Copy Number analysis more amenable to non-purification, one-step lysis buffers such as Lyse-N-Go™.

Therefore consideration of cell type and number should be a factor in the choice of DNA extraction method to address the presence of potentially inhibitory histological dyes when using LMD.

Mixture Separation Study

The primary goal of this research was to develop a method for the pure cell separation from a sperm/epithelial mixture amenable to forensic STR analysis. Gill developed a preferential lysis method (Gill et al., 1985), which was subsequently modified (Yoshida el al., 1995) becoming the 'gold standard' for separation of sperm from victim DNA in sexual assault cases. However, this method is often beset by incomplete separation resulting in female DNA contaminating the sperm fraction and the recovery of sperm is often reduced due to premature lysis and multiple sample manipulations. The forensic research community has continued to investigate alternative improved methods for cell separation including flow cytometry by florescence-activated cell sorting (Schoell el al., 1999), microchip-based sperm and epithelial cell separation (Horseman el al., 2005), and membrane filtration (Chen el al., 1998).

Laser microdissection has several potential advantages over previous separation methods. It requires only minimal manipulation of the sample and works by direct microscopic visualization, making it suitable for minute quantities of sperm. The first generation of this technology was termed laser capture microdissection (LCM) **(**Arcturus Bioscience, Mountain View, CA). LCM technology involves the use of a laser to microscopically melt a thermoplastic film onto a target cell embedding and lifting the cell from the slide. This technology has been used to recover sperm from microscope slides

(Elliott el al., 2003). While LCM allows the recovery of an enriched sperm fraction, female carryover can be relatively common from cell mixtures (Elliott el al., 2003). Carryover can be due to female DNA from lysed cells adhering to the sperm (Spadafora 1998). Alternatively it could be due to the non-specific attachment of surrounding cells to the plastic membrane. Despite transfer of female DNA in the male fraction, LCM performs significantly better than the preferential lysis method in its ability of separate sperm from vaginal epithelial cells (Elliott el al., 2003).

More recent advances in LMD methodology as used in this study allow a more precise dissection of cells. In addition, sample recovery can be verified visually in a post-collection mode allowing the user to microscopically inspect the cells collected. Thus LMD can clarify cell source attribution of any genetic profile obtained.

STR results from this study demonstrate that pure populations of sperm are recovered from semen/epithelial cell mixtures and amplifications at higher cycle numbers further show the absence of any female DNA in the sperm fraction. The data did show a loss in yield associated using the LMD method, which is further addressed in the last section of this report. Nevertheless, using standard PCR conditions genotyping can be obtained from 75-300 sperm cells with most heterozygous peak ratios above 70%, an acceptable industry standard (Applied Biosystems 1998). Samples exhibiting peak height ratios below 70% were most likely due to low amounts of haploid cells and the presence of PCR inhibitors.

The technique of performing PCR for six additional cycles on the PCR product amplified first for 28 cycles was used in this study primarily to detect any potential female contamination in the sperm fraction. However, it also increased signal intensity

providing full Profiler Plus genotypes from the "75" and "150" sperm cell samples when allelic-drop out or partial profiles were observed under standard PCR from the same samples. Increasing PCR cycles above the optimized range can cause preferential amplification of one allele (Gill 2001) which was evidenced in lower overall peak height ratios observed in the "75 sperm" samples. Nevertheless, the preferential amplification did not affect the accurate and complete genotyping of the samples tested. This suggests a potential of LMD in recovering sperm fewer than 75 cells for low copy number analysis.

Conclusions

The results of this study demonstrate that laser microdissection is an effective technique for recovering spermatozoa from a sperm/epithelial cell mixture for standard forensic STR analysis. LMD collects pure populations of sperm with no apparent cross contamination from buccal epithelial cells. Either hematoxylin/eosin or nuclear fast red staining can be effectively used for sperm identification in conjunction with LMD separation for STR genotyping. Used in combination with the Lyse-N-Go™ extraction procedure the LMD method is a simple, low-manipulation method for the analysis of sperm cells.

Acknowledgements

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Laser Microdissection Recovery and Separation of Minute Numbers of Spermatozoa from Epithelial Cells for Low Copy Number STR

Introduction

An area of research of great interest in the forensic community is the capacity of obtaining genotypes from minute amounts of DNA. Low copy number (LCN) DNA analysis typically refers to the testing of less than ~100pg of input DNA into a PCR reaction and is a technique sensitive enough to analyze just a few cells (Gill et al., 2000). STR typing results have even been obtained from a little as a single buccal cell (Findlay et al., 1997). One of the popular methods to increase sensitivity for LCN analysis is to increase the number of PCR cycles. There have now been several cases where this has obtained profiles from cases involving minute amounts of DNA such as transfer of assailant epithelial cells after strangulation (Wiegand et al., 1997) and DNA from grips of tools (Van Hoofstat et al., 1998). One of the challenges of LCN analysis is generating a reliable DNA profile as this type of analysis typically is accompanied by artifacts such as allele ‘drop-in’, allele ‘dropout’, increased stutter products, and heterozygote peak imbalance (Butler 2005). These artifacts can even further complicate the analysis when de-convoluting genuine DNA mixtures and in deciphering if the observed peaks are PCR related or true alleles in biological samples from either single or multiple sources. In addition, the technique is so sensitive that exogenous contaminating DNA unrelated to a crime such as transfer of DNA from previous handlers of an object in question or unintentional transfer of DNA from technicians may be detected.

LMD technology has the capability of collecting pure populations of sperm cells from mixtures as described in the first section of this report. In addition to excluding

contaminating cells in a sample, LMD may also be useful for avoiding free floating exogenous DNA in a sample which is problematic in LCN work. In this study, LMD was tested for the recovery of minute numbers of sperm cells from mixtures for LCN DNA analysis. A series of three experiments were performed to assess the functionality of LMD collection for LCN analysis. First, the minimum number of LMD cells necessary for STR typing was determined using our LCN protocols. Second, a comparative study was performed on the LMD method vs. the preferential lysis method for the separation of sperm from increasing proportions of female epithelial cells for LCN analysis. Finally, four sexual assault case studies were examined using LMD and LCN analysis.

Materials and Methods

The materials and methods used for the Low Copy Number experiments are as described previously with the following addition and/or modifications:

Hemacytometry

A hemacytometer was used on some samples to determine the concentration of cells in the working solutions. A Bright Line counting chamber (Hausser Scientific) was used with 5µl of epithelial cell working solutions mixed with 5µl of nuclear fast red or 5µl of semen working solutions mixed with 4.5µl of nuclear fast red and 0.5µl of picroindigocarmine. Average values of four 1mm square frames were used to estimate cell concentrations of the working solutions using the following formula: 1µl = 1 cubic

mm = (# cells counted per square mm) X (dilution) X (10). The dilution factor for all samples was 2x to account for the staining solution.

Differential Extraction

Samples in the preferential lysis study were subjected to the differential extraction followed by phenol-chloroform purification (Gill et al., 1985) and performed by the Northeastern Illinois Regional Crime Laboratory.

PCR Conditions for Low Copy Number Analysis

DNA Amplification was performed using the AmpF*l*STR® Profiler Plus Kit™ (Applied Biosystems, Foster City, CA) under the following reaction conditions: 21µl AmpFlSTR PCR Reaction Mix, 1µl AmpliTaq Gold® DNA Polymerase LD, 11µl AmpFlSTR Profiler Plus Primer Set, 20µl sample DNA; Thermal cycling conditions - Incubate 95ºC for 11 minutes (polymerase activation); 94ºC for 1 minute (denaturation), 59ºC for 1 minute (annealing), 72ºC for 1 minute (extension) for 34 cycles; then 60ºC for 45 minutes (final extension). In samples where 38 PCR cycles were used PCR was performed as follows: 25µl of PCR product amplified under the 34 cycle conditions were removed and added to a new tube with 0.25µl of AmpliTaq Gold® DNA Polymerase LD, then PCR performed for four additional cycles as above.

Studies

Minimum Cell Number Study - Five mixed cell samples from 10 donors (5 semen and 5 oral swabs) were examined. Mixtures of buccal and sperm cells were created to be

equivalent to one oral swab and 1µl of semen. Collections of 5, 10, 20, 40 and 80 sperm cells stained with NFR were separated by LMD from each mixture. All samples were processed using Lyse-N-Go™ DNA isolation applying both 34 and 38 cycle PCR.

Preferential Lysis and LMD Separation Comparison - Ten donor samples (5 semen and 5 oral swabs) were examined by hemacytometry to determine cell concentrations. Mixtures of sperm and epithelial cells were created at ratios of 1:5, 1:10, 1:20, 1:40, 1:80 and 1:160 (sperm:epithelial) for a total of 30 samples. For the comparative study 25µl of each mixture was subjected to a preferential lysis with organic extraction while 9µl were prepared onto PEN slides for LMD separation. In the LMD samples 20-30 sperm cells were collected from each mixture followed by Lyse-N-Go™ DNA isolation. All DNA extracts were amplified for 34 cycles.

Case Studies - Four case study samples containing spermatozoa were obtained from the Northeastern Illinois Regional Crime Laboratory. These sexual assault cases were previously examined by the police laboratory using the preferential lysis method and in this study re-examined using the LMD method.

Results

Minimum Cell Number Study

To establish the minimum number of sperm cells detected by STR amplification under our LCN analysis conditions, sperm cells were recovered from mixtures of semen and female buccal cells followed by DNA analysis. An example of STR plots recovered

from a semen/buccal mixture is illustrated in figure 11 from various amounts of sperm cells. Under 34-cycle conditions all samples containing 80 sperm resulted in the detection of all 10 loci from the sperm donors with all electropherogram peaks above 200 RFU. In samples containing 40 sperm, an average of 97.8±1.4% of the male donors' alleles were detected with all peaks above 100 RFU, and in samples containing 20 sperm cells 93.6+3.1% of the male donors' alleles were detected with all but one detected allele peak above 100 RFU. A significant decrease in the detection of the male donors' alleles was observed in collections of 10 and 5 sperm cells ($p < 0.05$, ANOVA Bonferroni post hoc test). Samples containing 10 cells resulted in the detection of 71.8±8.4% of the male donors' alleles, while samples containing 5 sperm cells displayed 56.2±4.5% of male donors' alleles.

After using 4 additional PCR cycles (38 cycles), allele detection of the male donor did not significantly increase in samples containing 5 and 10 sperm cells. Data containing 20, 40 and 80 sperm were not included in this report due to the preponderance of peak heights above the linear range of the instrument and an abundance of PCR artifacts typical of increased PCR cycles such as increased stutter and minus-A nucleotide products.

Profiles were also examined for any possible epithelial cell carryover from the female donor. Although 33 of the samples in this experiment set contained only male donor alleles there were 2 samples that clearly exhibited contaminating alleles from the female donor (Figure 12). These samples were traced back to a single mixture preparation. Reproducible results were obtained when the LMD prepared slide from this mixture was used for re-analysis (data not shown). However, when the mixture was re-

prepared from the original donor samples and analyzed no female carryover was observed in the LMD collected sperm fraction.

Comparative and Mixture Ratio Study

To further evaluate the separation and sensitivity of the LMD method, sperm from specific ratios of sperm/epithelial cell mixtures were separated using both the LMD and a preferential lysis method. STR plots from the sperm fractions from a 1:5 and 1:160 cell mixture are illustrated in figure 13 showing similar results with the 1:5 mixtures but dramatically different plots with the 1:160 mixtures. The percentages of male donor alleles detected from the samples are summarized in Figure 14. At a mixture ratio of 1:5 and 1:10 the allele detection of the sperm donor was near 100% for both methods. At the mixture ratios of 1:20 and 1:40 the allele detection for the LMD method remained constant at 99% but the average percentage of male alleles detected for the preferential lysis method fell below 80%. At a mixture ratio of 1:80 the allele detection under the LMD method was 100% whereas detection dropped to 57.4$\pm$9.4% ($p < 0.001$, ANOVA Bonferroni post hoc test) when the preferential lysis method was used. Finally, at a mixture of 1:160 the allele detection for the LMD and preferential lysis method was 97.8$\pm$1.4% and 56.6$\pm$8.3% respectively ($p < 0.001$, ANOVA Bonferroni post hoc test).

As a measure of PCR product quantity, total RFUs of the sperm donor profile was calculated from the STR data of the sperm fraction samples of both methods (Figure 15). Any non-shared female alleles were excluded in the analysis. At mixture ratios of 1:20, 1:40, 1:80 and 1:160 the LMD method produced significantly higher amounts of PCR product than the preferential lysis method ($p < 0.01$, ANOVA Bonferroni post hoc test).

Female DNA carryover into the sperm fraction was also compared by calculating the percentage of female alleles detected in the sperm fraction at each mixture ratio (Figure 16). Alleles shared by both donors were excluded from the analysis. Using the LMD method no female alleles were detected in any of the sperm fraction samples. However, using the preferential lysis method female donor alleles were detected in 12 of the 30 sperm fraction samples with DNA carryover frequency increasing as the proportion of epithelial cells increased.

Case Studies

STR results were obtained from all four cases and the data are summarized in table 4. Since the evidence had previously been analyzed by a police agency, a number of reported STR results were available including some exemplar profiles. It was our objective to recover 30 sperm cells from each sample; however, in one case only 18 sperm were identified and collected from a single slide.

Discussion

Minimum Cell Number

Lower limits of sensitivity recommended by manufacturers of STR multiplex systems are in the region of 250 pg (Gill 2001), which is equivalent to approximately 38 diploid or 76 haploid cells. We therefore chose to analyze groups of 5 -80 haploid sperm cells. This would be equivalent to the analysis of approximately 17-264pg of available DNA although based on the extraction yield information obtained in the last section of

this report the actual amount of isolated DNA available for PCR was most likely much lower than these values and well into the LCN analysis range. We had tested some different approaches to increasing the sensitivity for LCN analysis early in this research including experimenting with different commercial polymerases claiming to increase sensitivity and using reduced volume PCR amplification reactions (Gaines et al., 2002) (data not shown). However, we found that the technique of increasing PCR cycles (Whitaker et al., 2001, Kloosterman et al., 2003) was the simplest and most effective method for increasing the sensitivity for this project. A consequence of increasing PCR cycles is PCR artifacts become more frequent and pronounced. Allelic imbalance was common in these samples, which could occur due to preferential amplification of one allele or the loss in heterozygosity due to unequal sampling of just a few haploid sperm cells (Butler 2005). Allelic "drop-in" did not appear to be a problem with the samples we have discussed in this report but preliminary tests with LMD samples of only one or two sperm cells resulted in many non-specific amplification products and no clear peaks from the male donor (data not shown). Despite the above described artifacts the lower limit of sensitivity to obtain at or near full profiles was with 20 sperm cells, and clear partial profiles could still be obtained from as little as 5 cells demonstrating a high level of sensitivity.

Accompanying an assay with a highly level of sensitivity allows the opportunity of detecting unwanted contaminating DNA. There was one mixture that resulted in carryover of epithelial cell DNA into the sperm fraction of two LMD collected samples. Re-testing indicated that it was not a technician error but inherent to that particular mixture preparation. However, the exact cause of the carryover was not determined. We

did consider this a rare event, as carryover contamination did not occur in any other experiments in the entire course of this project even under higher PCR cycles. We felt this outlier was worth noting, as additional studies into circumstances that may create this problem would be important given the variety of environmental conditions surrounding forensic specimens.

Our aim to determine if recovery of cells by LMD could assist in analysis of low number of cells for LCN work was successful by just an increased PCR cycle approach to increase sensitivity. We expect that the detection of genetic markers could be further improved beyond that described here if other LCN analysis methods were used such as reduced volume PCR, introducing more PCR product into electrophoresis and, purification of PCR product (Budowle et al., 2001) or the use of more robust targets. There are also newer areas of research in the areas of whole genome amplification (Ballantyne et al., 2006) and more robust targets such as miniSTRs (Coble el al., 2005) or mtDNA (Coble et al., 2006) where laser microdissection could be a valuable cell recovery tool to accompany these techniques.

Comparative and Mixture Ratio Study

Spermatozoa and epithelial cells can be segregated based on their various properties. The most popularly used method to separate these cells in forensic sexual assault today is the preferential lysis method which takes advantage of the different cell properties to enzymatically digest the cell membranes of epithelial cells without lysing sperm cells (Gill et al., 1985). Although, this method is limited by some loss of sperm during initial digestion which can be particularly problematic when few are available in

the sample. Also the incomplete removal of epithelial cell DNA from the sperm fraction can occur especially if there is a high concentration of epithelial cells in the sample further jeopardizing the genotyping of the male alleles. The LMD method of separation proposed in this study has a quite different approach in that it takes advantage of the cytologic differences in cells to distinguish spermatozoa from epithelial cells.

We were able to show that separation of sperm from epithelial cells for LCN analysis yields superior STR results compared to the preferential lysis method particularly from samples with a low sperm to epithelial cell ratios. LMD was consistent with producing near full profiles of the male donor while excluding any detectable female DNA from the sperm fraction up to the 1:160 mixture ratio tested. Although the STR results obtained from the preferential lysis method were similar to the LMD method when the sperm to epithelial cell ratio was at 1:5 or 1:10, the PL method's ability to separate and detect the male component began to diminish at the 1:20 ratio with dramatically poorer results as the epithelial proportions increased. In addition, the amount of PCR product produced from sperm recovered by LMD was significantly greater than the sperm fraction component of the PL method despite that 30 or fewer sperm were collected for each LMD sample. This advancement may be crucial when there are only a few sperm detectable after a sexual assault. The LMD technique described has the potential to advance the practice of sexual assault investigation in providing DNA identification of an assailant in more difficult physical evidence cases.

The LMD method also requires fewer steps and less time for complete analysis than the PL method when processing a single sample as illustrated in Figure 17. The preferential lysis separation and DNA quantification steps are eliminated reducing

manipulation of sample and the potential for contamination. Using the LMD method also is more likely to result in a single source profile which requires less time for the scientist to perform the data interpretation and reporting, than if a mixed genotype was obtained.

Case Studies

The samples tested in this project thus far were designed to mimic sexual assault type evidence and were of clinical quality. This was necessary to maintain a controlled and measurable study. With the success of these experiments it became clear that the final test of the LMD method would be to examine real sexual assault evidence samples. Forensic specimens are unique in that they are exposed to an unlimited variety of conditions that may impact the extent and quality DNA results. Degradation of DNA can occur rapidly in a warm and/or moist environment, which is typical of the vaginal cavity, the most likely location to recover rape evidence. In addition, the highly acidic chemistry of the vagina may further speed up the breakdown of sperm. Along with degradation is the deteriation of cell structure in forensic samples. Characteristic morphologic landmarks and staining patterns used in clinical samples may not be as well defined or consistent in forensic specimens exposed to a variety of environmental insults. This is of particular interest due to the necessary cytological identification component required of the LMD method.

Here we tested portions of four sperm positive evidence samples with the assistance of LMD and LCN analysis. In case “A” a male was observed masturbating in a public facility, and tissue paper was found at the scene. In each of the unrelated cases “B”, “C” & “D” there was an alleged male sexual assault of a female where vaginal

swabs were collected. No further information was available regarding the details of the crimes including the post-coital interval to collection, age or storage conditions of the samples.

Case A was believed to be a neat semen sample and unlikely to be a mixture. In addition, no obvious epithelial cells were observed during LMD sperm collection. Results show a likely full male profile but no suspect exemplar profile was available for comparison. However, our results matched the alleles initially reported by the investigating laboratory.

The three vaginal swabs obtained in this study proved to be quite challenging. The cell pellets were recovered and prepared onto PEN slides; however, during the sperm examination insufficient numbers of sperm were recovered due to either the inability to locate any sperm on the slide or a high density of vaginal epithelial cells hindering identification of sufficient numbers of sperm for DNA analysis. Therefore we applied a quick mild digest approach in an attempt to remove some of the epithelial cells. A portion of the cell pellet sample was processed through a "mini-PL" consisting of a 30-minute first digest followed by a single wash. This cleaner cell pellet was then prepared onto a PEN slide. We found that this resulted in the quick identification of sufficient numbers of sperm for LMD recovery. This suggests a potential problem with LMD separation in casework samples containing high densities of epithelial cells or debris that can make identification of the spermatozoa difficult. A possible solution to alleviate this problem may be to dilute the cell pellet and smear the sample over a larger surface area of the slide. Unfortunately, the available amount of sample was limited in this study and there was insufficient amount of material to perform additional testing.

We tested only three vaginal swabs and it is important that a much larger sample set consisting of a broad representation of case samples be examined. This type of examination can better define criteria for case samples that may benefit from LMD separation over the traditional PL method. We recommend that a public forensic laboratory, where the availability and variety of case samples are in abundance, conduct this task.

Despite the modification of the LMD protocol required to obtain results these cases did show that in casework samples sperm can be recovered and successfully typed when collected by laser microdissection. As shown in table 4, the sperm from case B was consistent with a single source profile matching the suspect exemplar genotypes, and excluded the victim as a contributor. The crime laboratory's original results were consistent with a mixture of DNA from victim and suspect; therefore, the LMD method better resolved the sperm donor in case B. In case C a full male profile was obtained that excluded the victim, whereas, previously the crime laboratory obtained only a partial male profile. Our results provided more information in case C to better identify the sperm donor, however, it is important to note that we used LCN analysis to boost sensitivity which was not used in the original investigation. Finally in case D, we detected a partial profile with severe allelic dropout in which the victim could be eliminated as a contributor. Unlike the previous case, the crime laboratory's results for case D had less allelic dropout therefore revealing more of the sperm donor profile.

In summary, of the four cases tested our results produced similar results in one case, improved results in two cases and one case provided less information than when originally tested by the crime laboratory.

Conclusions

The results of this study demonstrate that laser microdissection is an effective technique for recovering and separating spermatozoa from a sperm/epithelial cell mixture for low copy number analysis. STR results can be obtained with minute amounts of sperm cells and therefore can facilitate the analysis of low numbers of sperm cells. Since epithelial cell carryover was extremely rare in our study, cross contamination is unlikely and the LMD technique is an excellent tool in obtaining a clean separation. The comparative study showed how the LMD method outperforms the PL method in detection and separation for low numbers of sperm as epithelial cell proportions in mixtures increase, which could lead to improvements on success rate of difficult cases. The visual nature of the collection process also adds confidence in cell source attribution of STR profiles, which is of particular interest in low copy number analysis. Genuine case samples may prove to be a challenge for LMD processing given the difficulty we encountered in the limited case samples tested at the conclusion of our research. However, given the small sample set examined our difficulties may not best represent routine casework in a forensic run laboratory. Therefore, a much larger examination of casework will be required including possible sample preparation modifications to make a final evaluation of the LMD method for forensic casework use.

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DNA Yield Evaluation from Laser Microdissected Cells

Introduction

Determination of the amount of DNA in a sample is important for most PCR-based assays including the Profiler Plus™ multiplex kits used in this study and in many forensic applications. In addition the DNA Advisory Board standards (DAB 2000) indicate an estimate be made of the amount of human DNA recovered from evidence samples. Although there are several methods that may be used to quantify DNA (Butler 2005) for forensic casework all require the consumption of part of the evidence sample, which may pose a problem either with conserving sample for re-analysis or in cases where only low amounts of DNA are present. The LMD collection process may provide an indirect estimate of DNA obtained without consuming any additional DNA extract. Since the number of cells can be counted during LMD collection the amount of starting DNA material in the cells can be calculated. However, loss of DNA during the isolation procedure must also be considered and some measure of the extraction efficiency can then be used to estimate the amount of DNA in the final extract prior to PCR. In this study we performed quantitative analysis on DNA extracts from LMD cells to determine the amount of loss associated with the extraction process and the feasibility of using LMD collection as a quantitative measure of DNA for PCR.

Materials and Methods

Quantification of human DNA was performed to estimate the DNA yield from cells collected using the LMD method. Two methods were used to evaluate yield. The first was by direct quantification of DNA by a real-time qPCR. The second method was using total RFU signal of STR results to measure relative amounts of PCR product to a standard curve.

Samples from 10 donors (5 semen and 5 buccal swabs) were examined. Each single source sample was prepared on a PEN slide and stained with NFR. Collections of 5, 10, 20, 40, 80, 150, and 300 sperm cells were recovered by LMD from the semen slides. Collections of 2, 5, 10, 20, 40, 80, and 150 epithelial cells were collected by LMD from the buccal cell slides. All samples were extracted using QIAamp® isolation. A dilution series of DNA standards with known quantities was also processed through the extraction procedure. Extracts were quantified using a real-time Alu-based qPCR method (Nicklas et al., 2003) with the following modifications: 1) Total reaction volume was increased to 20µl maintaining the proportions of reaction components and 2) real-time PCR was performed on a Bio-Rad iCycler (Bio-Rad Laboratories, Hercules, CA). In addition, portions of the DNA extracts from the sperm cell samples were amplified and analyzed for AmpF*l*STR® Profiler Plus loci at 34 PCR cycles using ½ volume reactions (25µl) while maintaining the same reagent proportions as the full volume reactions described earlier.

Results

To determine the amount of DNA loss associated with either the extraction procedure or LMD process both sperm and epithelial cells were examined using our methods. Results from the epithelial and sperm cell collections are summarized in Table 5. A reduction of yield was considered if the measured value of the test sample was lower than the expected value. The expected value assumed that the theoretically available DNA contained in the cells was retained. When DNA quantity was measured by qPCR, the average yield from the epithelial cell samples was 39.4 $\pm$ 1.5% whereas yields for sperm cell samples were significantly lower at 16.5$\pm$1.4% ($p<0.001$, ANOVA Bonferroni post hoc test). When DNA quantity was estimated using

florescent signal of STR products as a quantitative measure, the average DNA yield for the sperm samples was 22.7±1.1% , significantly greater than when qPCR was used ($p<0.05$ ANOVA Bonferroni post hoc test). Finally, the series of known DNA quantities that were re-extracted through the QIAamp® procedure were further quantified by both qPCR and by STR product resulting in a 13.0±2.0% and 27.5±4.7% yield respectively.

Discussion

Quantification of DNA is typically performed prior to PCR in forensic samples such that an optimal amount of DNA can be added into the reaction. This avoids the addition of too much DNA which may result in off-scale data for the instrumentation used (Butler 2005) and the exaggeration of stutter products. When collecting cells with laser microdissection one can preemptively avoid the addition of an overage of DNA into the reaction as the method itself allows the collection of precise numbers of cells. In addition it would be laborious to collect an overabundance of cells beyond what is necessary for analysis. Quantification can also assist in determining if there are sufficient amount of DNA for STR typing. Although low levels of DNA does not preclude performing PCR, an analyst aware that a sample contains low amounts of DNA can be better prepared for STR data exhibiting allelic-drop out and may even modify the route of analysis, such as using concentration techniques, increasing PCR cycles or electrophoresis injection time. Since the number of cells collected in each sample is known using the LMD method the DNA in the starting material collected can be estimated assuming each diploid cell contains 6.6pg of DNA or each haploid cell contains 3.3pg. We expected some loss of DNA during processing of the sample which was first observed in the mixture separation study discussed earlier in this report. This prompted us to further determine overall yield of

DNA recovered from the original cells collected when using our methods. In addition, we sought to determine if the extraction process was the primary source of DNA loss.

Some challenges arose in quantifying the samples collected by LMD. The DNA extracts in this study contained very low levels of DNA from about ½ ng down to only a few picograms in an entire extract. The current popular methods used to quantify forensic samples such as QuantiBlot® and Quantifiler™ (Applied Biosystem, Foster City, CA) were not sensitive enough to detect DNA at the lower levels in our study. We did however find that the Alu-based qPCR method (Nicklas et al., 2003) was sensitive enough to detect our LMD samples. A second problem arose when the DNA extraction method used was not compatible with this qPCR assay. The DNA isolation study discussed earlier in this report showed that Lyse-N-Go™ extraction with DTT was a rapid, simple, effective and relatively low cost method to extract DNA from sperm cells. We therefore continued to use this method throughout our research on LMD collected sperm cells. However, we found that the extraction reagents had an inhibitory effect on the qPCR reaction despite that no inhibition was observed when Lyse-N-Go™ extracts were amplified using the AmpF*l*STR® reaction. Several efforts at removing the inhibitory components from the extracts were made including removal of DTT and exchanging the extract into a neutral buffer solution, however, some level of either inhibition remained or the additional sample manipulations created opportunities for further sample loss. We did determine that the QIAamp® extraction method did not have any observed inhibitory effect (data not shown) and therefore used this method for this yield evaluation experiment for both sperm and epithelial collected LMD cells.

The results show that the amounts of DNA recovered from the cells were substantially lower than what the starting material contained. The highest yields of DNA were from the

epithelial cells where approximately 1/2 to 1/3 of the DNA was recovered, but DNA from sperm cells was consistently much lower as less than 1/4 of the DNA was obtained. It is unknown why the extraction efficiency differed between the two cell types. To determine if the extraction process was the likely cause of the low recovery of DNA, a dilution series of a known quantity of DNA was processed through the QIAamp® procedure and then re-quantified. Like the sperm cell samples, these DNA standards exhibited a similar loss in yield after the extraction process. This indicates that the DNA loss is occurring during the extraction process and not due to a failure of LMD cell recovery into the collection tube. What is important in this experiment is that the efficiency of the QIAamp® extraction remained relatively consistent across a range of starting material amounts obtained by LMD. Therefore, during the collection process LMD can also be used to estimate the quantity of DNA available for PCR.

Conclusions

An advantage of using the LMD method, whether it is for mixture separation or single source cell recovery, is that laborious intermediary DNA quantification analysis can be eliminated. Instead, cells can easily be counted during LMD collection, starting DNA material calculated, and then final DNA quantity can be estimated factoring in extraction efficiency prior to PCR. Despite the low extraction efficiencies reducing the available amount of DNA for PCR, STR results can still consistently be obtained from 300 sperm to as little as 5 sperm cells as demonstrated by both this and previous experiments in this report using standard and increased cycles PCR.

Figures and Tables

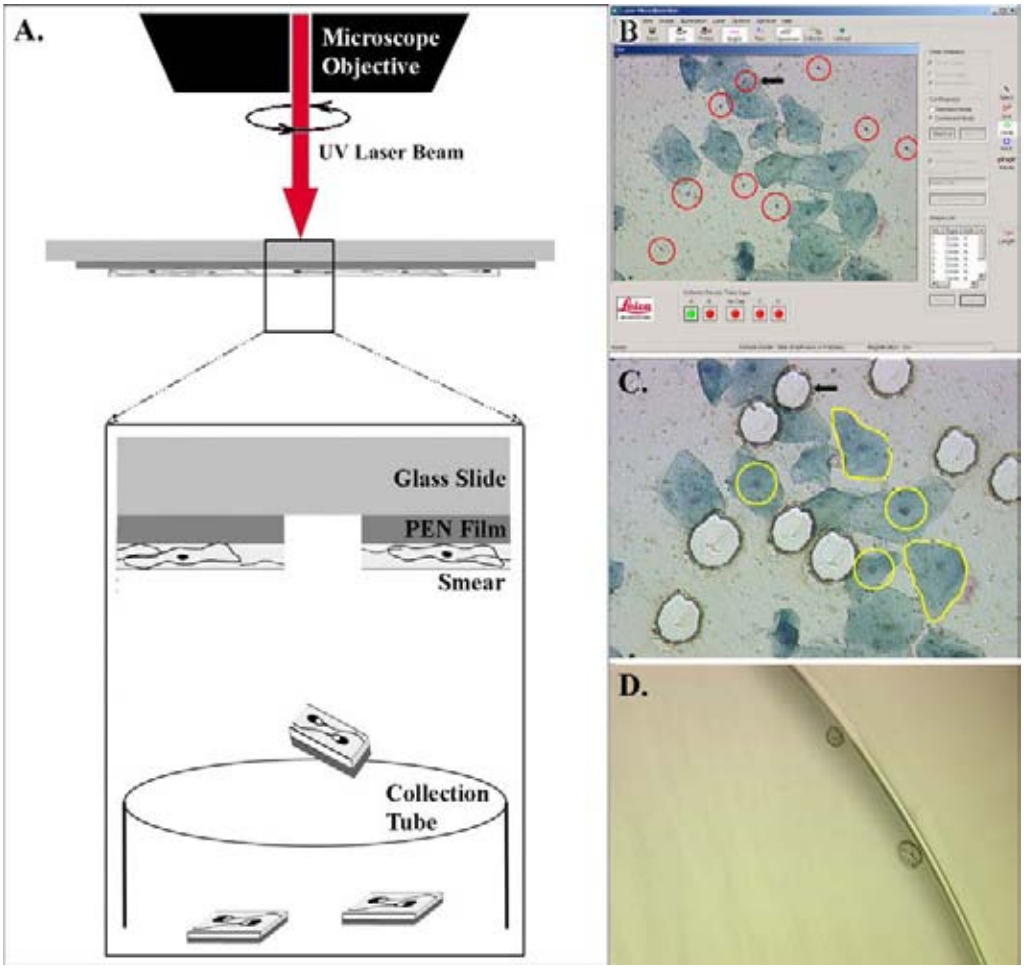

FIG. 1- *Laser Microdissection on the Leica AS LMD A) The plastic covered glass slide rests inverted on the microscope stage with the cell smear facing down. The laser is focused through the objective to dissect around sperm cells, cutting the PEN film. Magnified cross section illustrates sperm cells adhered to PEN film dropping into collection tube cap directly below stage. B) Leica AS LMD collection software window (version 4.1.3) with operator drawn circles (in red) around sperm cells. C) After laser microdissection of sperm cells, cuts can be verified (example indicated by black arrow) and epithelial cells are targeted by operator for dissection (in yellow). D) Post-collection feature of instrument allows inspection of collection tube to confirm recovery of sample.*

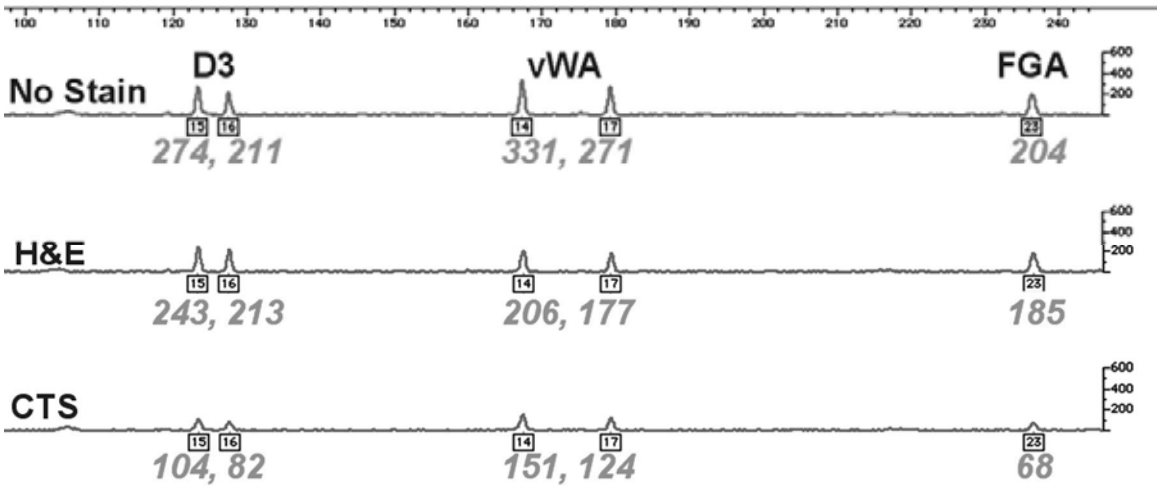

FIG. 2- *Peak height comparison of histologically stained cells. Examples of STR Plots at the D351358, wWA and FGA loci from 150 oral epithelial cells with no stain, H&E and CTS. Italicized numbers below plot are RFU values of each peak.*

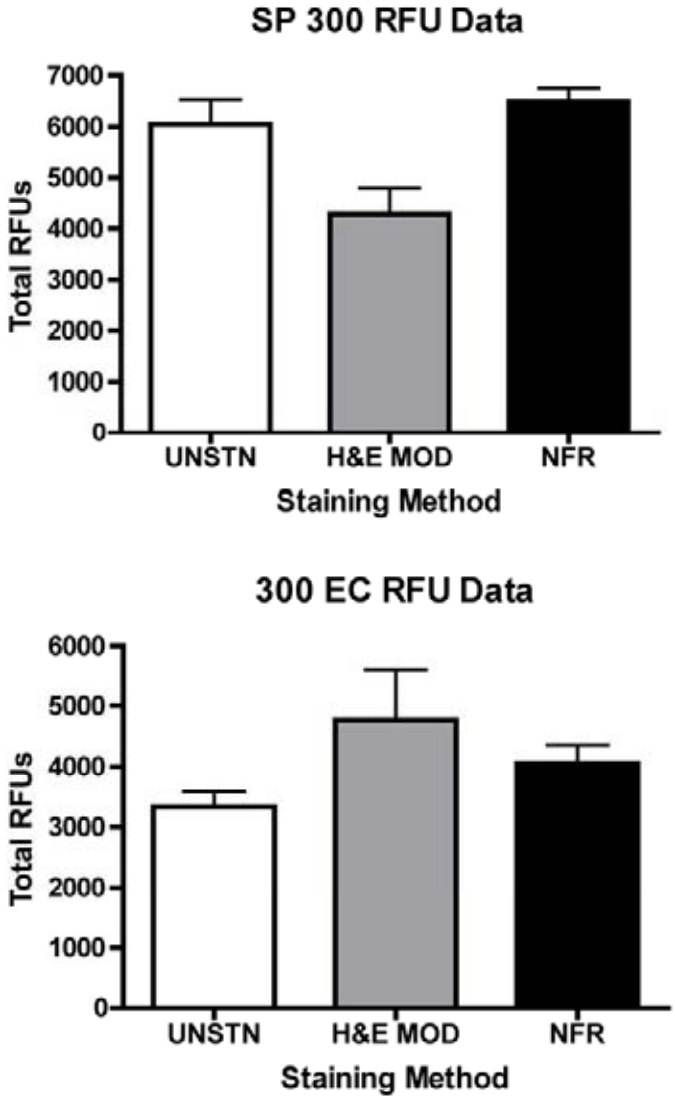

FIG. 3 – Quantitative comparison of PCR product from stained and unstained cells in Group 2 study. Samples comprising of 300 sperm and 300 epithelial cells were stained with modified H&E or nuclear fast red. Mean total RFU values (n = 5) from Profiler Plus amplifications are shown in each group.

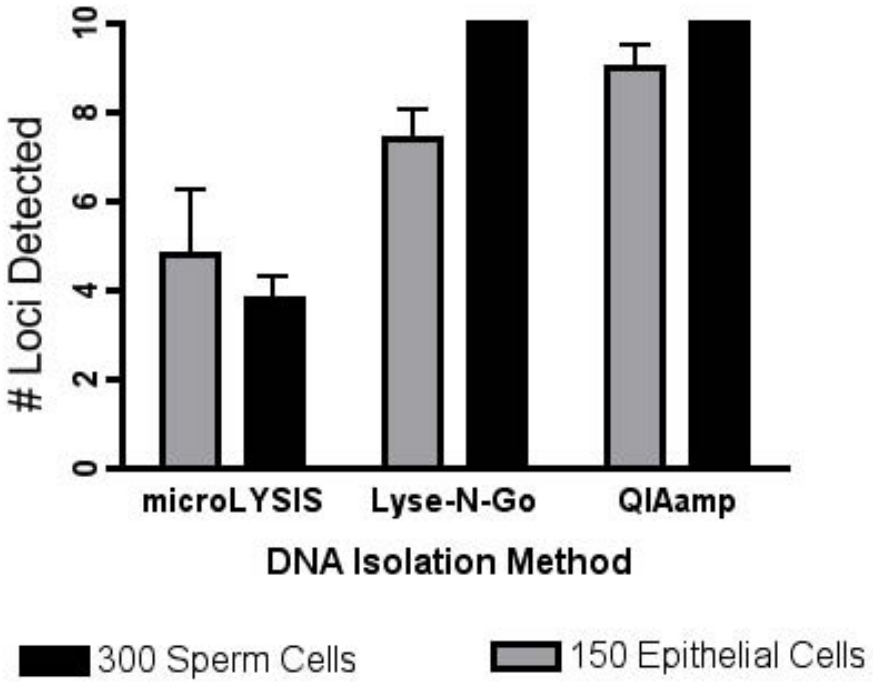

FIG. 4- *Detection of STR loci using different isolation methods. Samples comprising of 300 sperm and 150 oral epithelial cells were subjected to three DNA isolation methods: microLYSIS®, Lyse-N-Go™ and QIAamp®. Mean number of loci (n=5) detected out of ten possible Profiler Plus markers is shown from each group.*

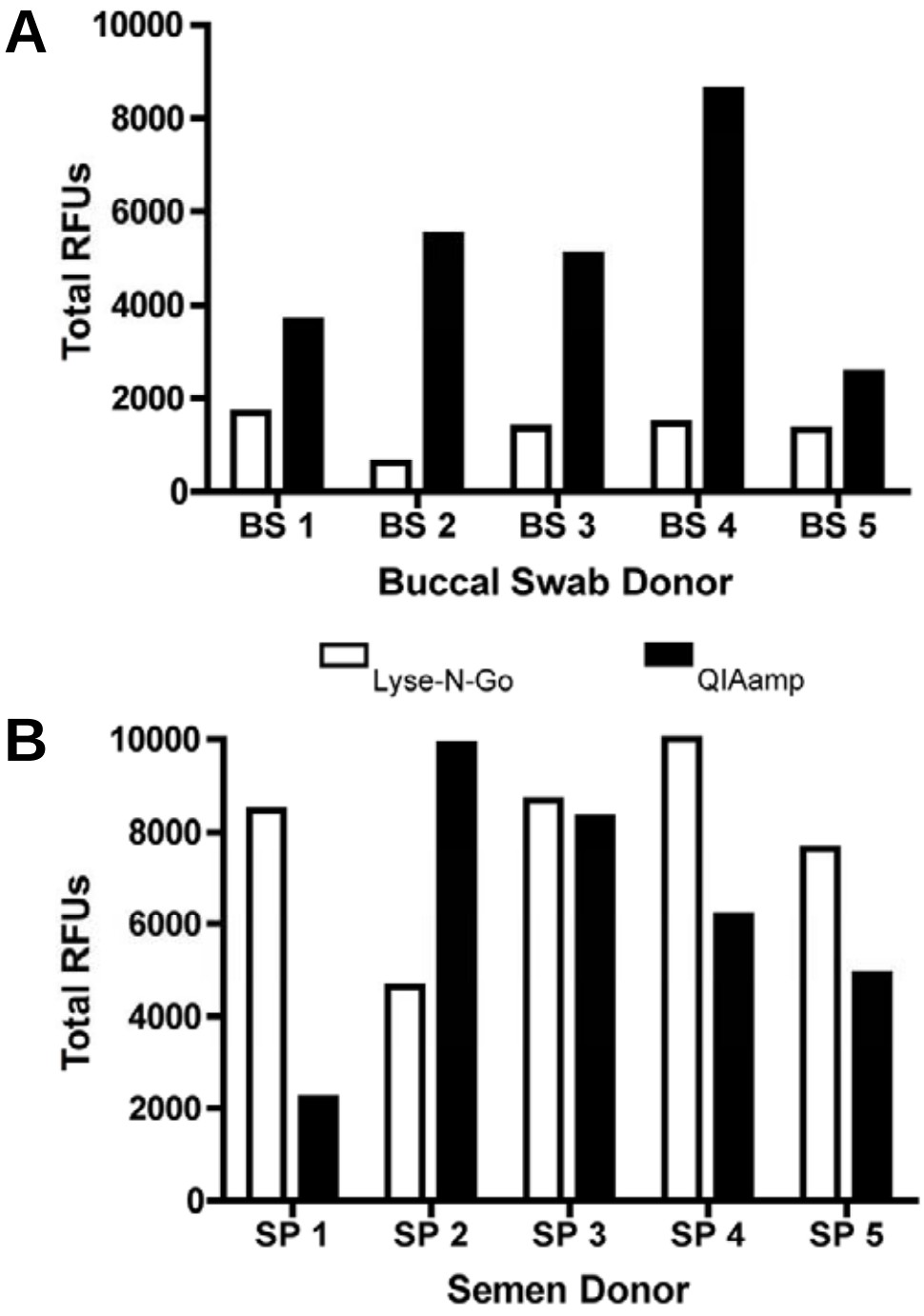

FIG. 5 – *Total PCR product detected from LMD collected cells using Lyse-N-Go and QIAamp DNA isolation methods. A) 150 epithelial cells each from five buccal swab donors B) 300 sperm cells each from five semen donors.*

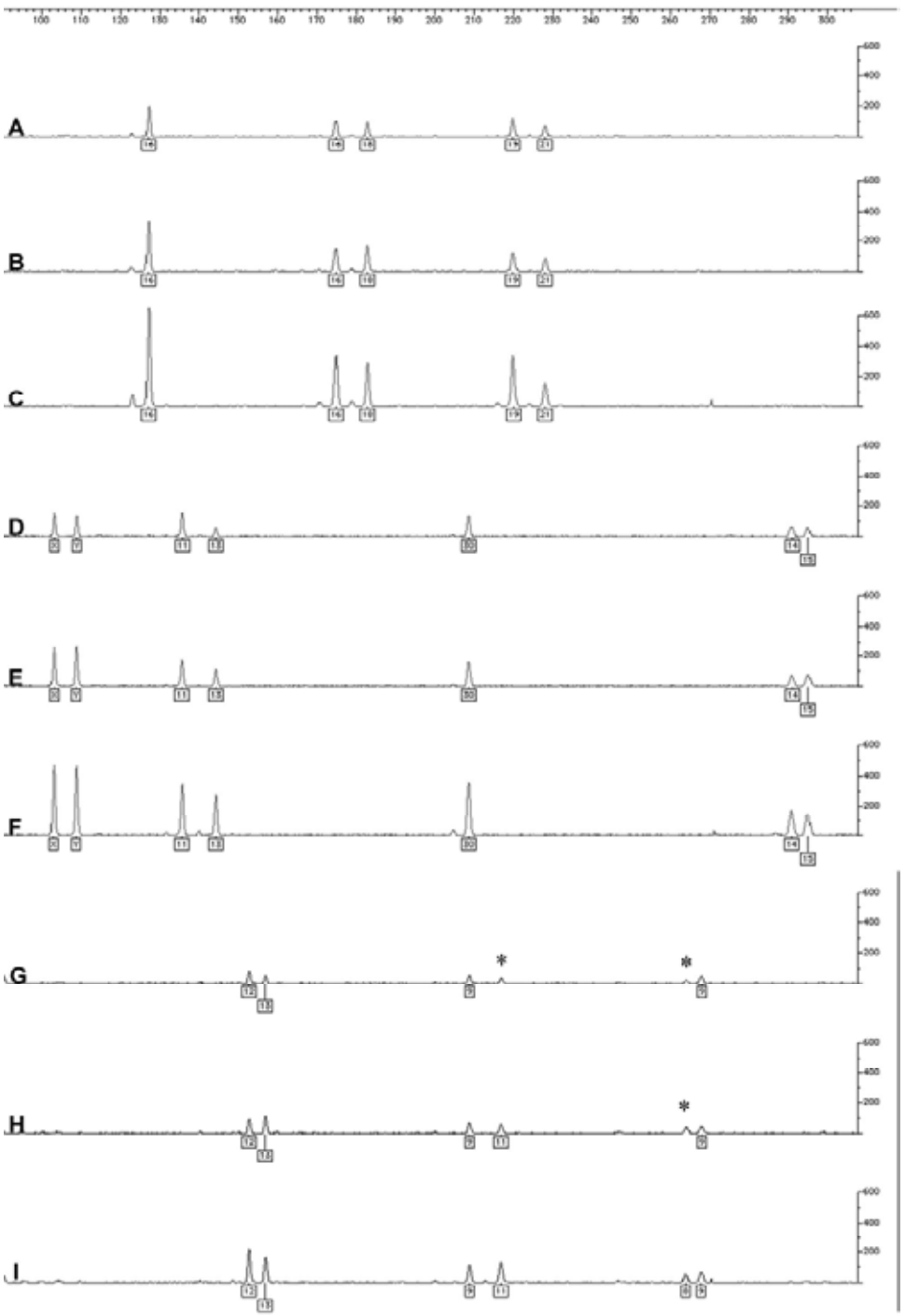

FIG. 6- *Profile Plus plots of sperm separated by LMD from a mixture. Blue loci of A) 75 sperm B) 150 sperm and C) 300 sperm. Green loci of D) 75 sperm E) 150 sperm and F) 300 sperm. Yellow loci of G) 75 sperm H) 150 sperm and I) 300 sperm (y-scale at 600 RFU). Allelic drop-out observed in yellow loci for 75 and 150 cells indicated with an asterisk.*

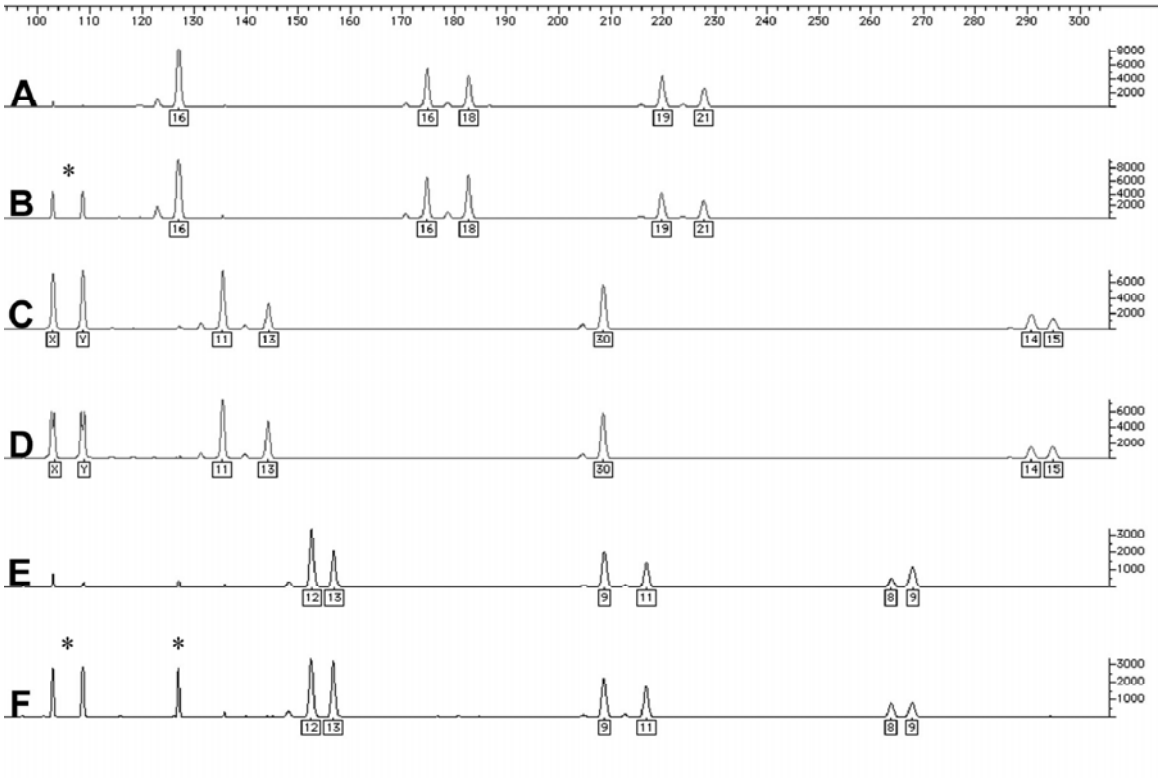

FIG. 7- *Profiler Plus plots of LMD collected sperm cells from a sperm/epithelial cell mixture using extended cycles PCR. Blue loci of A) 75 sperm B) 150 sperm. Green loci of C) 75 sperm D) 150 sperm. Yellow loci of E) 75 sperm F) 150 sperm. All alleles detected from male donor without female carryover. Notable spectral pull-up observed from "150 sperm" samples indicated with an asterisk.*

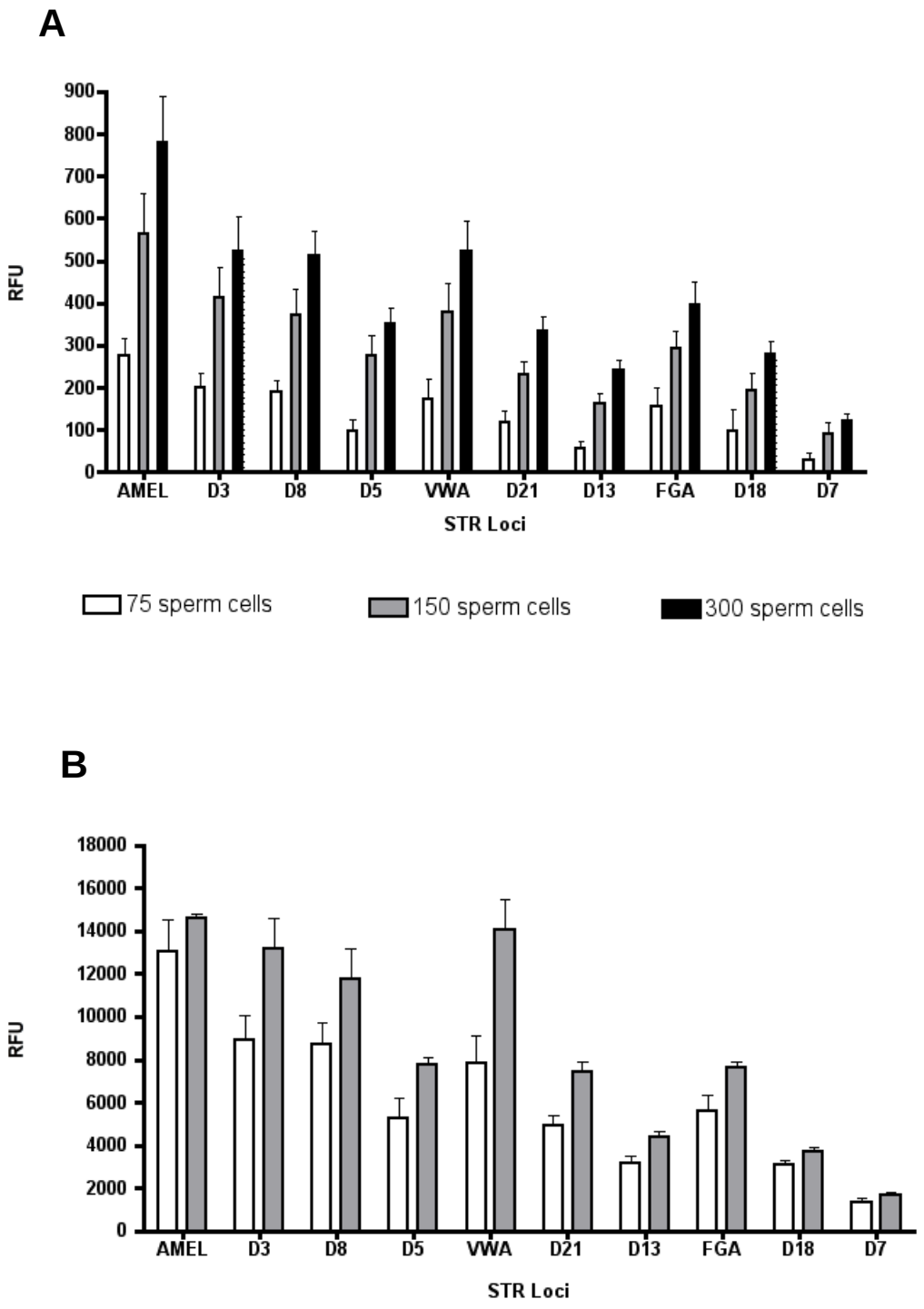

FIG. 8- *Fluorescent signal at each locus for standard and extended cycles PCR. Total RFU peak values at each locus were averaged for A) 75, 150, and 300 sperm cell specimens using standard PCR conditions and B) 75 and 150 sperm cell specimens using extended cycles PCR.*

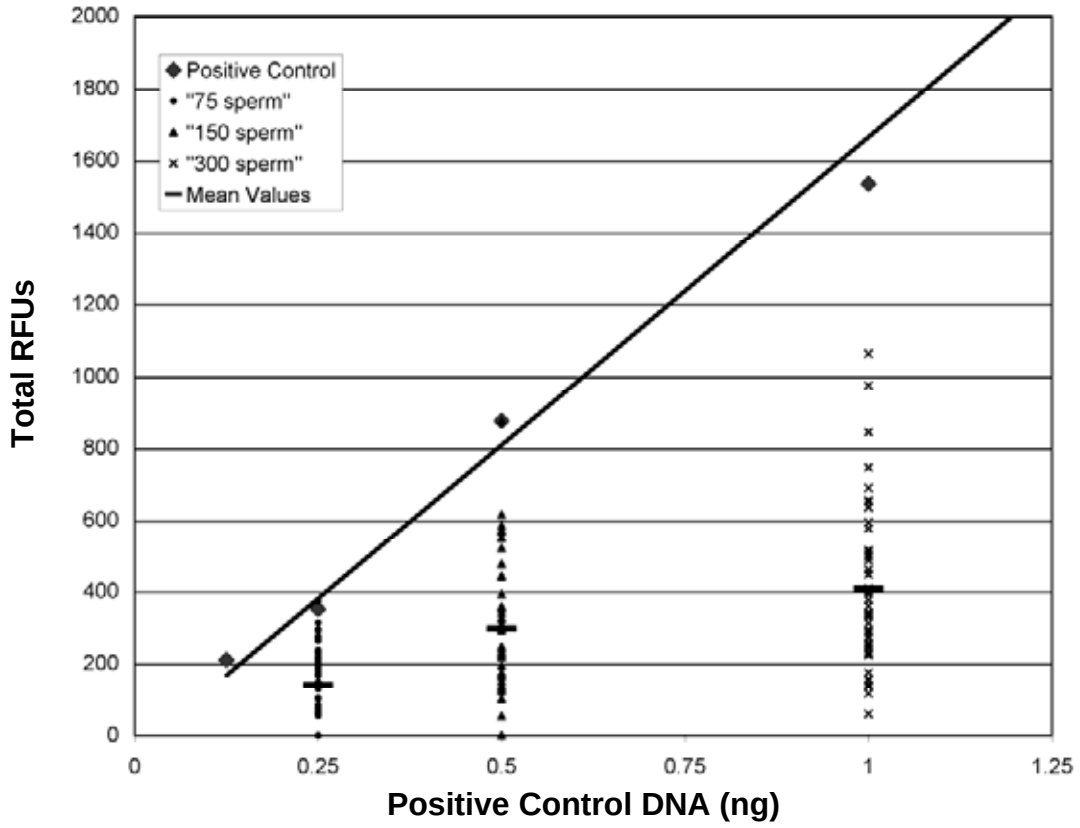

FIG. 9- *PCR product level of 75, 150, and 300 cells under standard PCR conditions. Five positive control dilutions of 0.125, 0.25, 0.5, 1.0 & 2.0ng of Human DNA (2.0ng not shown) plotted against observed total RFU values. Total RFU values for 75, 150 & 300 sperm samples were plotted on the x-axis at the corresponding maximum theoretical DNA quantity.*

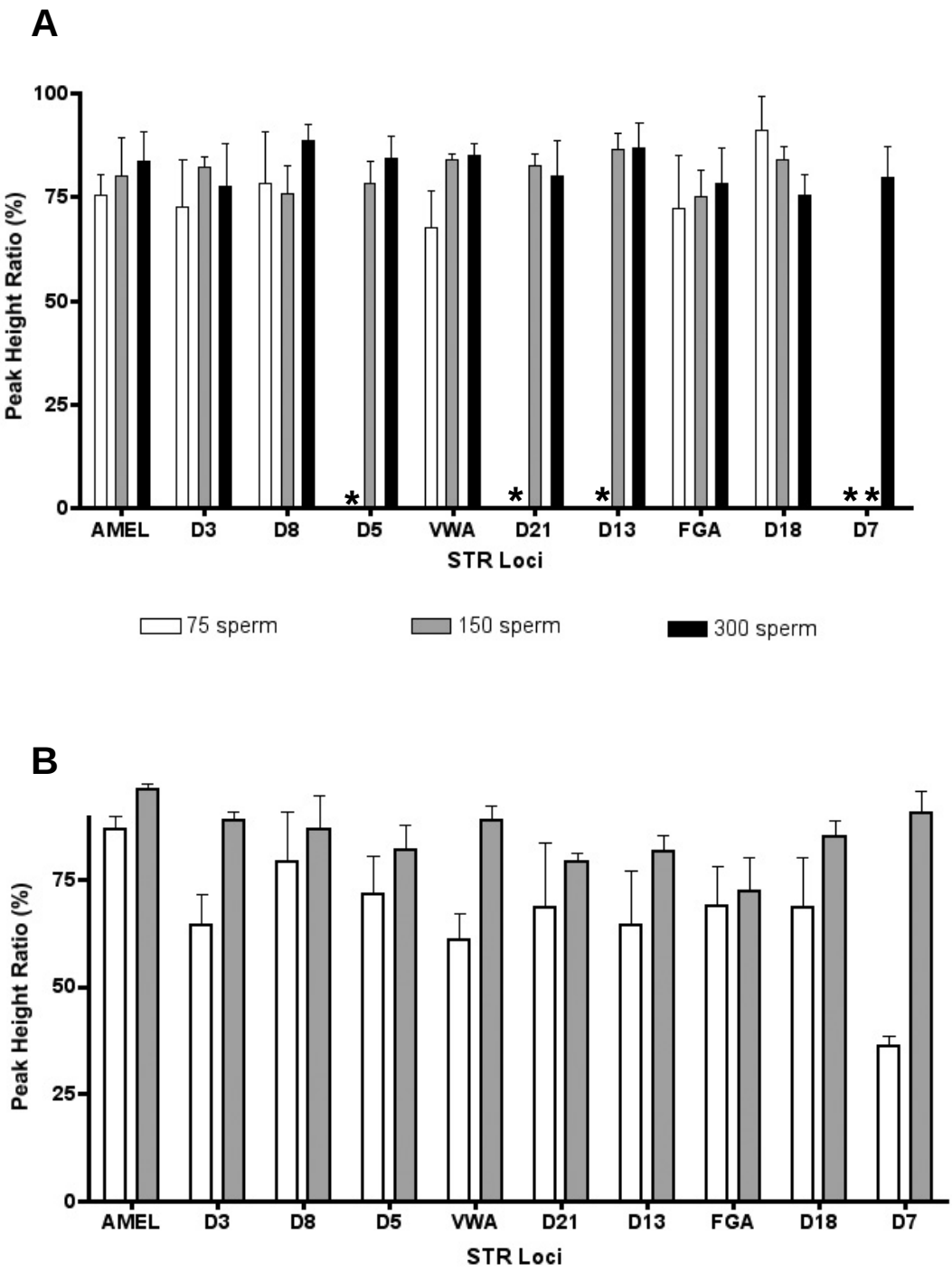

FIG. 10- *Peak height ratios at each locus for standard and extended cycles PCR. Peak height ratios of heterozygous loci were averaged for A) 75, 150, and 300 sperm cell specimens using standard PCR conditions and B) 75 and 150 sperm cell specimens using extended cycles PCR.*
** Insufficient heterozygous data for analysis*

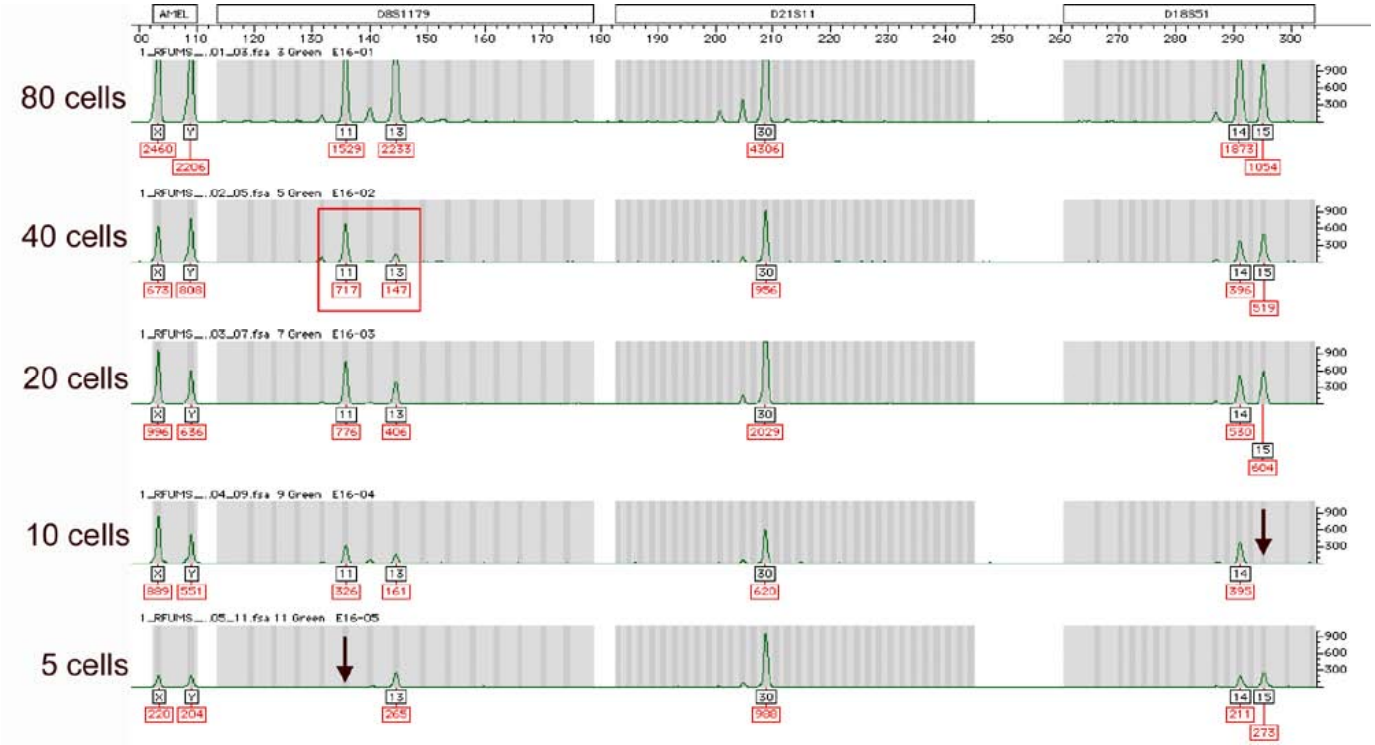

FIG 11- STR plots from sperm cells separated from a semen/epithelial cell mixture. Green loci shown from a Profiler Plus amplification of 5, 10, 20, 40 & 80 sperm cells at 34 PCR cycles. Red values indicate peak heights. Examples of allelic imbalance indicated by red box and allelic dropout by vertical arrows.

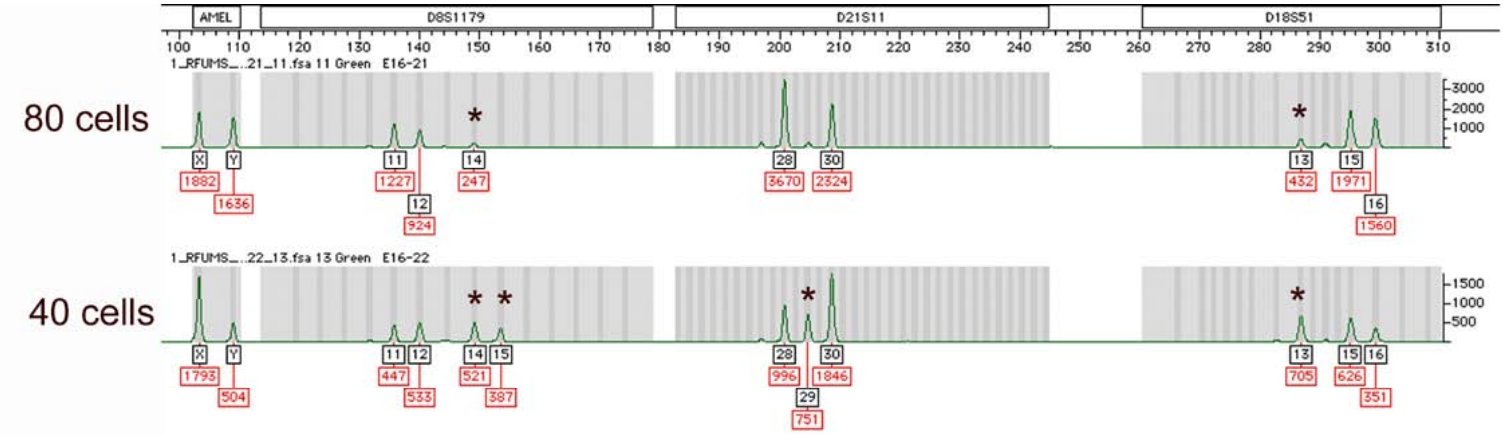

FIG. 12 – STR plots from LMD collected sperm cells with epithelial cell DNA carryover. Female donor alleles (indicated by asterisks) were detected in the 40 and 80 sperm cell collections from one of the slide smears in this study.

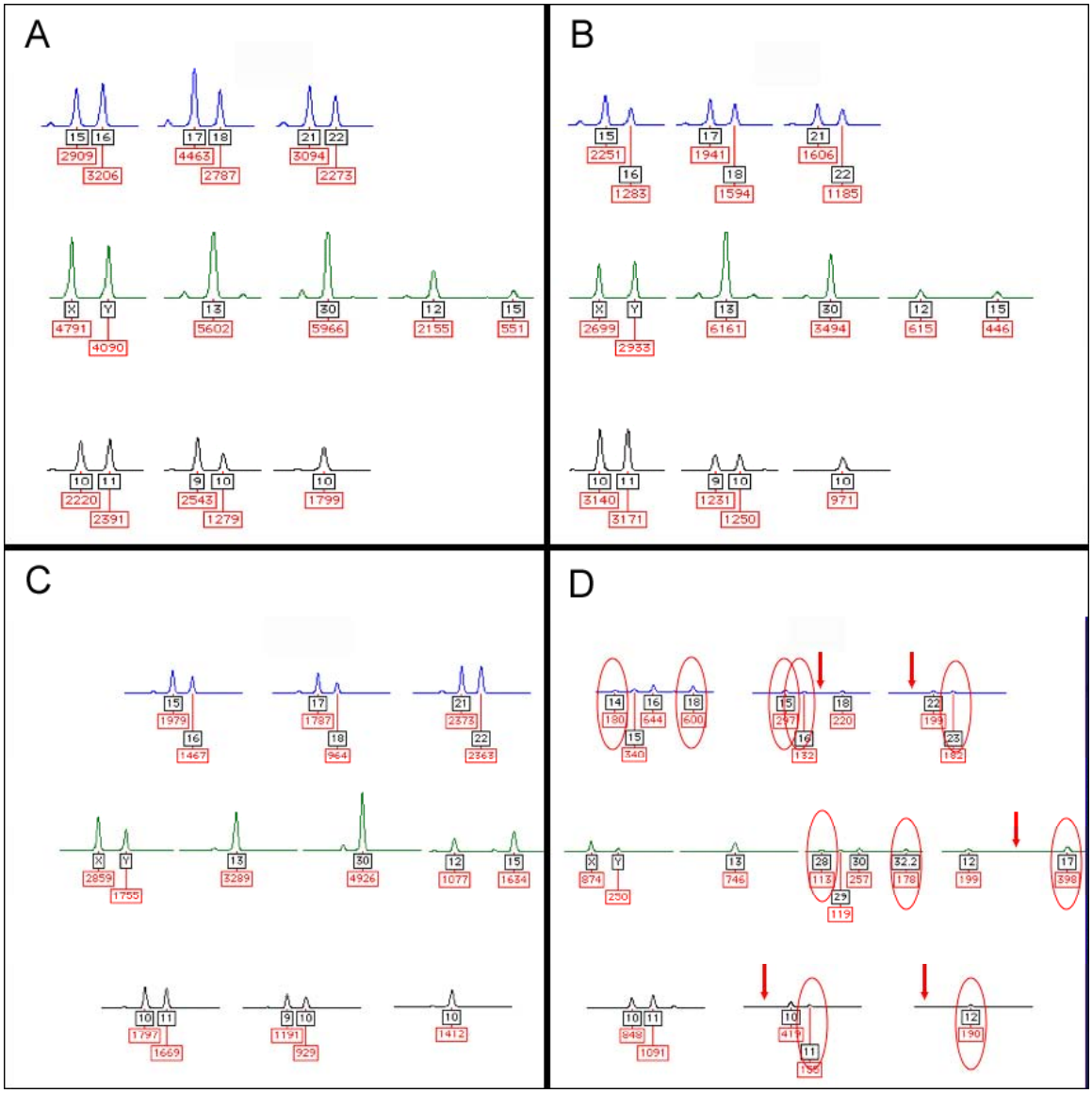

FIG. 13 – Comparative STR plots from sperm fractions using the LMD and preferential lysis methods. LMD separation from a A) 1:5 and C) 1:160 sperm to epithelial cell mixture; B) Preferential lysis separation from the same B) 1:5 and D) 1:160 sperm to epithelial cell mixture. RFU values are in red under peaks. Red circles indicate female donor DNA detected in the sperm fraction. Allelic dropout of the male donor alleles indicated by red arrows.

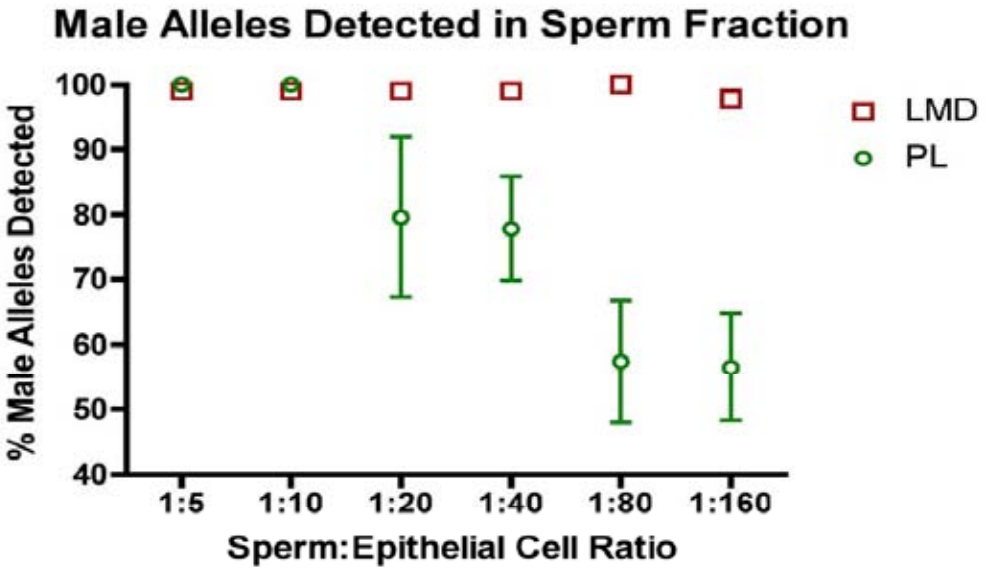

FIG. 14 – Comparison of the LMD and PL methods' ability to detect the male component. The percent of male alleles detected for each separation method was determined from 1:5, 1:10, 1:20, 1:40, 1:80 and 1:160 sperm to epithelial cell mixtures (n=5 for each category).

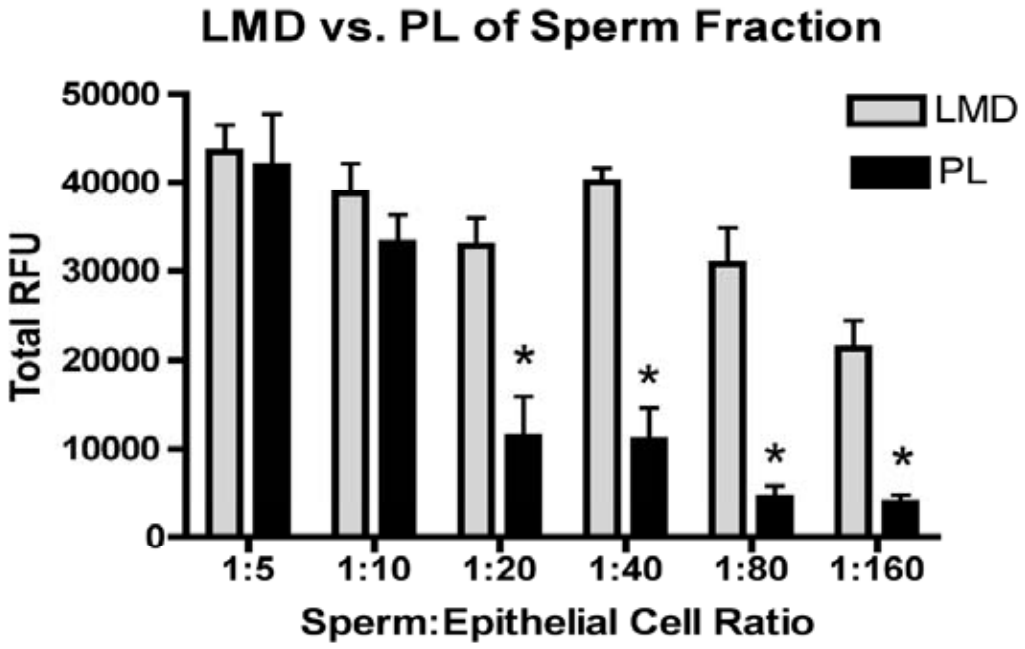

*FIG. 15 – Comparison of relative PCR product amounts from the sperm fractions after using the LMD and PL methods. Total RFU values from STR profiles is used as measure of PCR product produced after sperm separation from 1:5, 1:10, 1:20, 1:40, 1:80 and 1:160 sperm to epithelial cell mixtures. * = significantly lower amount observed ($p < 0.01$). Standard error bars represent n=5.*

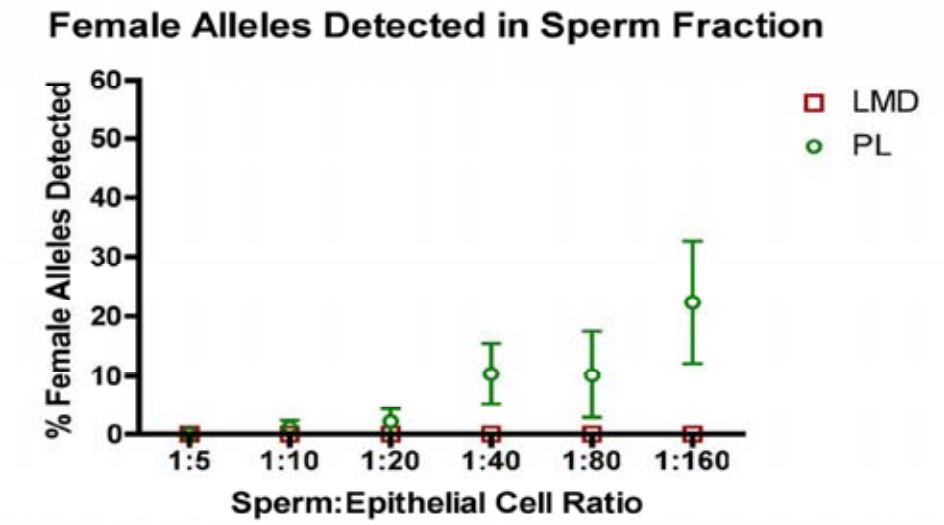

FIG. 16 - Comparison of the LMD and PL methods' ability to separate the male component. The percent of female alleles detected for each separation method was determined from 1:5, 1:10, 1:20, 1:40, 1:80 and 1:160 sperm to epithelial cell mixtures (n=5 for each category).

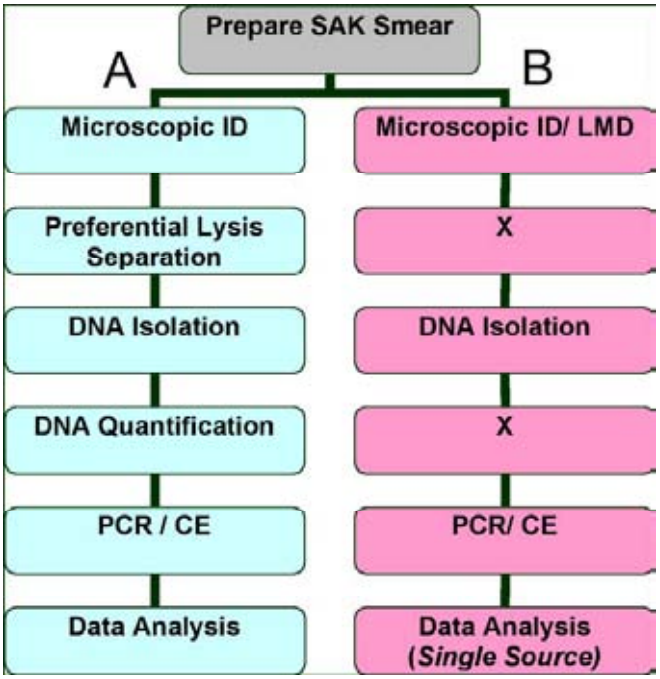

FIG. 17 – DNA Analysis flowchart for sperm evidence using A) preferential lysis separation or B) LMD separation.

TABLE 1 - *LMD Parameters for Sperm and Epithelial Cells using 40x Objective*

Parameter	Sperm cells with low epithelial cell density	Epithelial cells or Sperm cells with high epithelial cell density
Aperture	2/16	2/16
Intensity	39/46	44/46
Speed	6/20	3/20
Bridge (Gap)	Medium	Medium
Aperture diff	6	6
Off set	40	40

Denominators denote maximum setting allowed. Numerators denote setting used for collection

TABLE 2 – *Microscopic identification scores of sperm and epithelial cells for each histology stain.*

		Histological Stain					
	Sample	UNSTN	H&E	CTS	MG	WRT	AO
Spermatozoa	1	+ / -	+	+	- -	-	+
	2	+ / -	+	+ +	-	- -	+
	3	+ / -	+	+ +	- -	-	+
Buccal Cells	1	+ / -	+	+ +	-	- -	+ / -
	2	-	+	+ +	-	- -	-
	3	+ / -	+	+ +	- -	- -	+

UNSTN = not stained.
H&E = hematoxylin/eosin.
CTS = nuclear fast red/picroindigocarmine.
MG = methyl green.
WRT = Wright's stain.
AO = acridine orange.
- - : cannot ID or highly challenging
- : poor
+ / - : satisfactory
+ : good
+ + : excellent

TABLE 3 - *Number of male donor alleles detected from LMD sperm fraction.*

		# alleles detected ≥50RFU		
Sample	# expected alleles	"75 sperm"	"150 sperm"	"300 sperm"
1	18	16	17	18
2	19	18	19	19
3	19	13	19	19
4	17	5	17	17
5	20	16	17	20

TABLE 4 –*Profiler Plus genotypes obtained from case studies*

Locus	**Case A sp LMD**	**Case A sp original PL results**	**Case B sp LMD****	**Case B sp original PL results**	**Case B suspect**	**Case B victim**
D3S1358	16, 17	16, 17	17	15, 16, 17	16, 17	15, 16
vWA	17	17	15, 18	15, 16, 18	15, 18	15, 16
FGA	21, 23	21, 23	19, 24	19, 22, 24	19, 24	22, 25
AMEL	X, Y	X, Y	X, Y	X, Y	X, Y	X
D8S1179	12, 14	12, 14	10, 13	10, 13, 14	10, 13	14, 17
D21S11	28, 31	28, 31	30.2, 31	30, 30.2, 31	30.2, 31	30, 31.2
D18S51	18	18	15, 17	15, 16, 17	15, 17	16
D5S818	9, 12	9, 12	8, 12	8, 12, 13	8, 12	13
D13S317	11, 12	11, 12	12, 14	12, 14	12, 14	12
D7S820	8, 12	8, 12	9	8, 9, 10	9, 10	8, 12

Locus	**Case C sp LMD****	**Case C sp original PL results**	**Case C victim***	**Case D sp LMD****	**Case D sp original PL results**	**Case D victim***
D3S1358	15	15	16	15	15, 16	15
vWA	16, 18	16, 18	16	18	16, 18	17, 18
FGA	24, 25			24	24	
AMEL	X, Y	X, Y	X	X	X, Y	X
D8S1179	13, 15	13, 15	10, 14	12	12, 13	12, 13
D21S11	28, 31.2	28	30, 31.2	32.2	28, 32.2	28, 30
D18S51	14, 16			13		
D5S818	11, 12	11, 12	10, 11	10	10, 13	8, 11
D13S317	10, 13		9, 10	13	12, 13	8
D7S820	11, 12			10		

* Partial profile of victim assumed from the epithelial cell fraction of vaginal swab
** LMD performed after "mini-PL"
sp = sperm fraction

TABLE 5 – DNA yields of LMD collected cells following extraction

# cells	Theoretical amount of DNA in starting material* (pg)	% Yield by real-time qPCR	% Yield by STR RFUs
300 sperm (n=5)	990	**22.9** ± 4.8 %	Off scale data
150 sperm (n=5)	495	**13.3** ± 2.3 %	Off std. curve
80 sperm (n=5)	264	**16.3** ± 5.4 %	**25.1** ± 6.6%
40 sperm (n=5)	132	**18.5** ± 3.8 %	**22.0** ± 5.6%
20 sperm (n=5)	66	**12.2** ± 4.7 %	**20.1** ± 7.8%
10 sperm (n=5)	33	**14.8** ± 0.8 %	**23.6** ± 6.6%
5 sperm (n=4)	16.2	**17.8** ± 2.9 %	Off std. curve
150 epithelial (n=5)	990	**37.5** ± 2.8 %	
80 epithelial (n=5)	528	**45.4** ± 6.8 %	
40 epithelial (n=5)	264	**37.5** ± 4.2 %	
20 epithelial (n=5)	132	**40.5** ± 7.0 %	
10 epithelial (n=5)	66	**32.9** ± 5.3 %	
5 epithelial (n=5)	33	**41.4** ± 8.6 %	
2 epithelial (n=5)	13.2	**40.7** ± 11.0 %	
AB Pos control	31.3 -1,000	**13.0** ± 2.0 % (n=6)	**27.5** ± 4.7% (n=5)

* assuming each sperm cell contains 3.3 pg of DNA

References

- Allery JP, Telmon N, Mieusset R, Blanc A, Rouge D. Cytological detection of spermatozoa: comparison of three staining methods. J Forensic Sci 2001;46(2);349-51.
- Applied Biosystems. AmpFlSTR Proliler Plus user's manual. Foster City, CA: Applied Biosystems 1998.
- Ballantyne KN, van Oorschot RA, Mitchell RJ. Comparison of two whole genome amplification methods for STR genotyping of LCN and degraded DNA samples. Forensic Sci Int. 2006 May 8.
- Budowle B, Hobson DL, Smerick JB, and Smith J. Low copy number – consideration and caution. Proceedings of the 12th International Symposium on Human Identification. Madison, WI 2001
- Burton MP, Schneider BG, Brown R, Escamilla-Ponce N, Gully ML. Comparision of histologic stains for use in PCR analysis of microdissected, paraffin-embedded tissues. Biotechniques 1998 Jan;Vol. 24;1:86-92.
- Butler, J.M., Forensic DNA Typing. Burlington: Elsevier Academic Press, 2005.
- Butler, J.M., Forensic DNA Typing. London: Harcourt Science and Technology, 2001.
- CDC. Sexual Violence: Fact Sheet. Retrieved September 25th, 2006 from http://www.cdc.gov/ncipc/factsheets/svfacts.htm
- Chen J, Kobilinsky L, Wolosin D, Shaler R, Baum H. A physical method for separating spermatozoa from epithelial cells in sexual assault evidence. J Forensic Sci 1998 Jan;43(1):114-8.
- Coble, M.D. and Butler, J.M. (2005) Characterization of new miniSTR loci to aid analysis of degraded DNA. *J. Forensic Sci.* 50(1):43-53.
- Coble, M.D., Vallone, P.M., Just, R.S., Diegoli, T.M., Smith, B.C., Parsons, T.J. (2006) Effective strategies for forensic analysis in the mitochondrial DNA coding region. I*nt. J. Legal. Med.* 120:27-32.
- DNA Advisory Board, Quality Assurance Standards for Forensic DNA Testing Laboratories, Forensic Science Communications July 2000 vol 2 Number 3
- Ehrig T, Abdulkadir SA, Dintzis SM, Milbrandt J, Watson MA. Quantitative amplification of genomic DNA from histological tissue sections after staining with

nuclear dyes and laser capture microdissection. J Mol Diagn 2001 Feb;3(1):22-5.

- Elliott K, Hill DS, Lambert C, Burroughes TR, Gill P. Use of laser microdissection greatly improves the recovery of DNA from sperm on microscope slides. Forensic Science International 2003;137:28-36.

- Emmert-Buck MR, Bonner RF, Smith PD, Chuaqui RF, Zhuang Z, Goldstein SR et al. Laser capture microdissection. Science. 1996 Nov 8;274(5289):998-1001. Comment in: Science 1996 Nov 8;274(5289):921-2.

- Findlay I, Taylor A, Quirke P, Frazier R, Urquhart A. DNA fingerprinting from single cells. *Nature*. 1997 Oct 9; 389(6651): 555-6.

- Gaines ML, Wojtkiewicz PW, Valentine JA, Brown CL. Reduced volume PCR amplification reactions using the AmpFlSTR Profiler Plus kit. *J Forensic Sci* 2002; 47(6):1224-1237.

- Gill P, Jeffreys AJ, Werrett DJ. Forensic application of DNA 'fingerprints'. Nature 1985 Dec 12-18;318(6046):577-9.

- Gill P, Whitaker J, Flaxman C, Brown N, Buckleton J. An investigation of the rigor of interpretation rules for STRs derived from less than 100 pg of DNA. *Forensic Sci Int*. 2000 Jul 24; 112(1): 17-40.

- Gill P. Application of low copy number DNA profiling. Croat Med J 2001 Jun;42(3):229-32.

- Greenspoon SA, Scarpetta MA, Drayton ML, Turek SA. QIAamp spin columns as a method of DNA isolation for forensic casework. J Forensic Sci 1998 Sep;43(5):1024-30.

- Herr, JC. U.V.A researchers developing new forensic tool. Retrieved January 19, 2004, from http://www.healthsystem.virginia.edu/internet/news/Archives 01/forensic.cfm.

- Horseman KM, Barker SLR, Ferrance JP, Forrest KA, Koen KA, Landers JP. Separation of sperm and epithelial cells in a microfabricated device: potential application to forensic analysis of sexual assault evidence. Anal Chem 2005;77(3):742-9.

- Kayser, M., Caglia, A., Corach, D., Fretwell, N., Gehrig, G., Heidorn, F., Herrmann, S., Herzog, B., Hidding, M., Honda, K., Jobling, M., Krawczak, M., Leim, K., Meuser, S., Meyer, E., Oesterreich, W., Pandya, A., Parson, W., Penacino, G., Perez-Lezaun, A., Piccinini, A., Prinz, M., Schmitt, C., Schneider, PM., Szibor, R., Teifel-Greding, J., Weichhold, GM., de Knijff, P., and Roewer, L. Chromosome Y microsatellites: population genetic and evolutionary aspects**.** (1997a) *International Journal of Legal Medicine*, 110, 125-133, Appendix 141-149.

- Kloosterman AD, Kersbergen P. Efficacy and limits of genotyping low copy number (LCN) DNA samples by multiplex PCR of STR loci. J Soc Biol. 2003;197(4):351-9.

- Ladd C, Adamowicz MS, Bourke MT, Scherczinger CA, Lee HC. A systematic analysis of secondary DNA transfer. *J Forensic Sci*. 1999 Nov;44(6):1270-2.

- Ladd C, Lee HC, Yang N, Bieber FR. Interpretation of complex forensic DNA mixtures. *Croat Med J*. 2001 Jun; 42(3): 244-6.

- Larkin A, Harbison S. An improved method for STR analysis of bloodstained denim. Int J Legal Med 1999;112(6):388-90.

- Lerman LS. Structural considerations in the interaction of DNA and acridines. J Mol Biol 1961 Feb;3:18-30.

- Luzzi V, Holtschlag V, Watson MA. Expression profiling of ductal carcinoma in situ by laser capture microdissection and high-density oligonucleotide arrays. Am J Pathol 2001 Jun;158(6):2005-10.

- Martino D, Giuffre G, Staiti N, Simone A, Le Donne M, Saravo L. Single sperm cell isolation by laser microdissection. Forensic Sci Int 2004 Dec 2;146 Suppl:S151-3.

- Mercurio M, Silenieks T, Royds D. An evaluation of the acridine orange and Christmas tree stains for the identification of spermatozoa. Forensic Bulletin June 1997.

- Molecular Probes, LIVE/DEAD® Sperm Viability Kit (L-7011) Product Information Insert. 2001

- Moore J, Aros M, Steudel K, Cheng K. Fixation and decalcification of adult zebrafish for histological, immunocytochemical, and genotypic analysis. BioTechniques 2002 Feb;32:296-8.

- Moxon ER, Wills C. DNA microsatellites: agents of evolution? *Sci Am*. 1999 Jan; 280(1): 94-9.

- Murase T, Inagaki H, Eimoto T. Influence of histochemical and immunohistochemical stains on polymerase chain reaction. Mod Pathol 2000 Feb;13(2):147-51.

- Nicklas JA, Buel E . Development of an Alu-based, real-time PCR method for quantitation of human DNA in forensic samples, , Journal of Forensic Science, Vol 48, No 5, 936-944, 2003.

- Oppitz E. Eine neue Farbemethode zum Nachweis der Spermien bei Sittlichkeitsdelikten (A new colouring method for the determination of sperm cells in sexual assault cases). Arch Kriminol 1969;144:145-8.

- Prinz M, Boll K, Baum H, Shaler B. Multiplexing of Y chromosome specific STRs and performance for mixed samples. *Forensic Sci Int.* 1997 Mar 14;85(3):209-18.

- Rook MS, Delach SM, Deyneko G, Worlock A, Wolfe JL. Whole genome amplification of DNA from laser capture-microdissected tissue for high-throughput single nucleotide polymorphism and short tandem repeat genotyping. Am J Pathol 2004 Jan;164(1):23-33.

- Sanders CT, Peterson DA. Laser microdissection separation of cell mixtures for STR analysis. Oral Presentation, International Association of Forensic Sciences meeting, Hong Kong, 2005.

- Sanders CT, Peterson DA. Separation of spermatozoa and epithelial cell mixtures by laser microdissection for forensic DNA analysis. Oral Presentation, American Academy of Forensic Sciences meeting, Dallas, TX, 2004,

- Sanders CT, Reisenbigler EK, Peterson DA, Laser microdissection: low copy number analysis of sperm from mixtures. Oral Presentation, American Academy of Forensic Sciences, Seattle, WA, 2006.

- Sanders CT, Reisenbigler EK, Pfoser K, Peterson DA. Laser microdissection for low copy number analysis of sperm from mixtures. Oral Presentation 7th Annual DNA Grantees' Workshop, NIJ, Washington, DC, 2006.

- Sanders CT, Sanchez N, Ballantyne J, Peterson DA. Development of laser microdissection to separate histologically stained mixed cells for DNA analysis. Oral Presentaion, California Association of Criminalists meeting, Ventura, CA, 2004.

- Sanders CT, Sanchez N, Ballantyne J, Peterson DA. Laser microdissection separation of pure spermatozoa from epithelial cells for short tandem repeat analysis. J Forensic Sci. 2006 Jul;51(4):748-57.

- Sanders CT, Sanchez N, Ballantyne J, Peterson DA. Laser microdissection of sperm: stain, cut, separate, lyse, and STRs. Oral Presentation. Mid-Atlantic Association of Forensic Scientists meeting, Pittsburgh, PA, 2005.

- Sanders CT, Sanchez N, Ballantyne J, Peterson DA. STR analysis of laser microdissected cells for mixed stain samples. Oral Presentation, Louisiana Association of Forensic Scientists meeting, Baton Rouge, LA, 2004.

- Schoell WM, Klintschar M, Mirhashemi R, Pertl B. Separation of sperm and vaginal cells with flow cytometry for DNA typing after sexual assault. Obstet Gynecol 1999 Oct;94(4):623-7.

- Serth J, Kuczyk MA, Paeslack U, Lichtinghagen R, Jonas U. Quantitation of DNA extracted after micropreparation of cells from frozen and formalin-fixed tissue sections. Am J Pathol 2000 Apr;156(4):1189-96.

- Simone NL, Bonner RF, Gillespie JW, Emmert-Buck MR, Liotta LA. Laser-capture microdissection: opening the microscopic frontier to molecular analysis. Trends Genet 1998 Jul;14(7):272-6.

- Spadafora C. Sperm cells and foreign DNA: a controversial relation. Bioessays 1998 Nov;20(11):955-64.

- SWGDAM, Scientific Working Group on DNA Analysis Methods, Short Tandem Repeat Interpretation Guidelines, *Forensic Science Communications*, Vol. 2 , July 2000

- Van Dijk M, Rombout P, Dijkman H, Ruiter D, Bernsen M. Improved resolution by mounting of tissue sections for laser microdissection. *J Clin Pathol: Mol Pathol* 2003; 56: 240-243

- Van Hoofstat DE, Deforce DL, Hubert De Pauw IP, Van den Eeckhout EG.DNA typing of fingerprints using capillary electrophoresis: effect of dactyloscopic powders. Electrophoresis. 1999 Oct;20(14):2870-6.

- Walker HK, Hall WD, Hurst JW, editors. Clinical methods: the history, physical, and laboratory examinations. 3rd ed. Stoneham: Butterworth-Heinemann, 1990.

- Walsh PS, Metzger DA, Higuchi R. Chelex 100 as a medium for simple extraction of DNA for PCR-based typing from forensic material. Biotechniques. 1991 Apr;10(4):506-13.

- Whitaker JP, Cotton EA, Gill P. A comparison of the characteristics of profiles produced with the AMPFlSTR SGM Plus multiplex system for both standard and low copy number (LCN) STR DNA analysis. Forensic Sci Int. 2001 Dec 1;123(2-3):215-23.

- Wiegand P, Kleiber M. DNA typing of epithelial cells after strangulation. Int J Legal Med 1997;110:181-3.

- Wilson IG. Inhibition and facilitation of nucleic acid amplification. Appl Environ Microbiol 1997 Oct;63(10):3741-51.

- Yoshida K, Sekiguchi K, Mizuno N, Kasai K, Sakai I, Sato H, Seta S. The modified method of two-step differential extraction of sperm and vaginal epithelial cell DNA from vaginal fluid mixed with semen. Forensic Sci Int 1995 Mar 21;72(1):25-33.